MECHANISM OF ACTION OF THE FUNGICIDE COPPER OXINATE

By

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I. INTRODUCTION

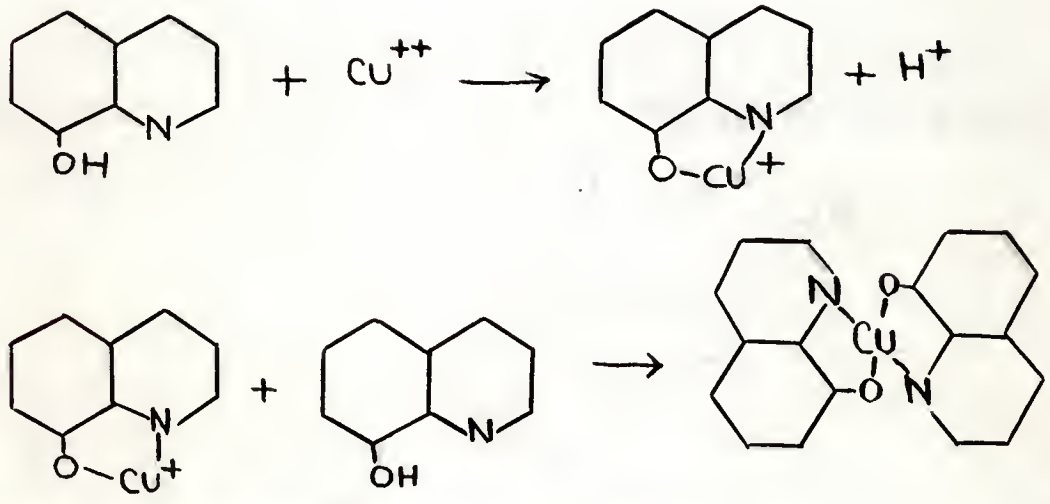
A. Literature Review

When chemical compounds show biological effects, such as toxicity to bacteria or fungi, they are subject to speculation and investigation as to their basis of action. In some cases the mode of action has been initially explained by an uncomplicated and apparently straightforward process, but found by further studies to be very complex. This is the case for 8-hydroxyquinoline.

Although 8-hydroxyquinoline has been used as a bactericide since the early nineteen hundreds, only recently has its mechanism of action been studied. The suggestion of Hata (1) that 8-hydroxyquinoline owes its toxicity to the presence of both a phenol and a quinoline portion combined in one molecule can not be seriously considered. On the basis of present evidence and because the toxicity of 8-hydroxyquinoline is more than fifteen times that of phenol and quinoline combined (2), this theory can not be considered valid.

Inasmuch as 8-hydroxyquinoline acts in very low concentractions (100 per cent inhibition of bacteria and

fungi at molar concentrations of 0.0001), it appeared likely that its action was involved in some metabolic process. From the consideration that 8-hydroxyquinoline could combine to form bery stable complex compounds, at biological hydrogen ion concentrations, with important ions such as cupric, ferrous, ferric, cobaltous, zinc, and manganic, Albert (3,4) and Zentmeyer (5,6) suggested independently that the action was occuring in the form of chelation between 8-hydroxyquinoline and the essential trace metals needed for healthy growth conditions. The complex formed between 8-hydroxyquinoline and copper may be represented as follows:



Albert used bacteria for his experimental work, while Zentmeyer employed fungi. The basic findings of their initial work were similar.

The toxicity of 8-hydroxyquinoline to bacteria, was investigated further by Albert and his co-workers (7). Of the seven possible monohydroxyquinolines, it was found that on blocking either of the groups essential for chelation, the oxygen and nitrogen in this case, the toxicity of the compound was lost along with its ability to chelate. When compounds were prepared which contained the characteristic groups of 8-hydroxyquinoline (a hydroxyl group peri to a ring nitrogen) in other nuclei such as 1-hydroxyacridine, 8-hydroxy-5:6-benzquinoline or 6-hydroxy-m-phenanthrolin, these compounds were found to possess considerable toxicity. Among the fifty analogues and derivatives of 8-hydroxyquinoline tested, only those such as 8-hydroxyquinoline-5-sulfonic, -5-carboxylic acids did not show the relationship between strong chelation and high toxicity. The two soluble acid derivatives chelate strongly but possess anionic groups that can prevent a molecule from penetrating the cell membrane. This fact supports the theory that the site of action is in the cell and not in the medium.

Zentmeyer offered evidence that 8-hydroxyquinoline inhibition was due to specific trace element immobilization for the fungi he studied. He showed first that 8-hydroxyquinoline did not inhibit the fungi below a pH 3, for this compound does not chelate well in strong

acidity. Second, that the toxic effect could be overcome by adding an excess of metal and third, increasing amounts of 8-hydroxyquinoline were required for inhibition in the presence of increasing concentrations of metal ions. Zentmeyer's work was carried out using only zinc in his studies. Zinc has been shown to be of lesser importance than copper or iron (7).

From these experiments it was obvious that 8-hydroxyquinoline was acting by chelation, but the site and the mechanism of action was not established. Albert and his coworkers have proposed a mode of action based on such experiments previously described and also on later work on the 8-hydroxyquinoline-copper equilibrium and the distribution of 8-hydroxyquinoline in a lipoid-water system (8,9). More recently, Vicklund, Manowitz and Bagdon have proposed a theory that suggests, on the basis of biological evidence, that the copper complex formed between 8-hydroxyquinoline and copper dissociates and the toxicity is due to the free 8-hydroxyquinoline complexing essential trace metals in the cells. They state that the copper ions liberated function synergistically with the 8-hydroxyquinoline (10). This theory can not be accepted on the basis of the work of Albert and the experiments to be described later.

From the unusual condition of low toxicity at high concentration (0.00125 molar) and high toxicity at low concentrations (0.00001 molar) of 8-hydroxyquinoline, Albert concluded that the toxic nature of this compound was related to the metals present in the medium. He proved this by depleting the medium through chelation of the metals and extraction of the chloroform soluble chelates. In this metal free-medium, 8-hydroxyquinoline was nontoxic toward the gram-positive bacteria used. The toxicity was rapidly restored by the addition of small amounts of ferric or cupric ions. From this it was concluded that the true toxic agents were the iron or copper chelates.

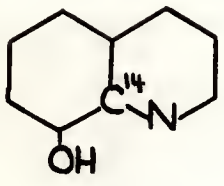
This conclusion was, however, only partially correct, for with excess amounts of 8-hydroxyquinoline and with proper trace metals present the expected toxicity was not found. If the 8-hydroxyquinoline concentration was decreased or the metal increased, the solution showed extreme toxicity. This indicated that the true toxic agent was a specific metal complex, namely, the 1:1 iron or copper 8-hydroxyquinoline complex. If a large excess of the trace metal was added the toxicity decreased. This was considered to be due to the formation of a 1:1 complex possessing either a single or double positive

charge, depending on the ion used, cupric or ferric. This charged complex would then be unable to enter the cell.

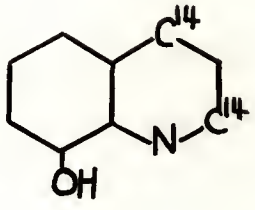
From this indirect type of information the mode of action of 8-hydroxyquinoline was thought to be one that probably occurred within the cell although no direct proof of the fact was available. For that matter, all evidence up to the present had been based mainly on indirect experimentation.

B. Statement of the Problem

The problem undertaken in this research was that of the establishment of a mechanism of action for the compound 8-hydroxyquinoline. The research reported here is concerned with the establishment of certain basic points, by direct methods such as using carbon-14 labeled compounds, necessary to either enhance or to change the hypothesis proposed by Albert and other workers referred to previously. It was necessary to prepare carbon-14 labeled 8-hydroxyquinoline for the first time. The carbon-14 was put in two positions. In one case it was in the angular position (I), using aniline-1-C^{14}, and in the other in the heterocyclic ring (II) using glycerol-1,-3-C^{14} as the carbon-14 source.



(I)



(II)

The most essential question to be answered was whether the 8-hydroxyquinoline was actually entering the cell or was it adsorbed on the cell wall. Once this

question was answered and thus the general site of action established inside the cell, the effects of various factors such as hydrogen ion, copper, and 8-hydroxyquinoline concentration on the fungus material could then be studied.

The problem involved further a study of the nature and properties of the copper complexes of 8-hydroxyquinoline. In summary, using carbon-14 labeled compounds as a direct method of investigation, the mechanism by which 8-hydroxyquinoline acts as a toxicant to fungi and bacteria was studied.

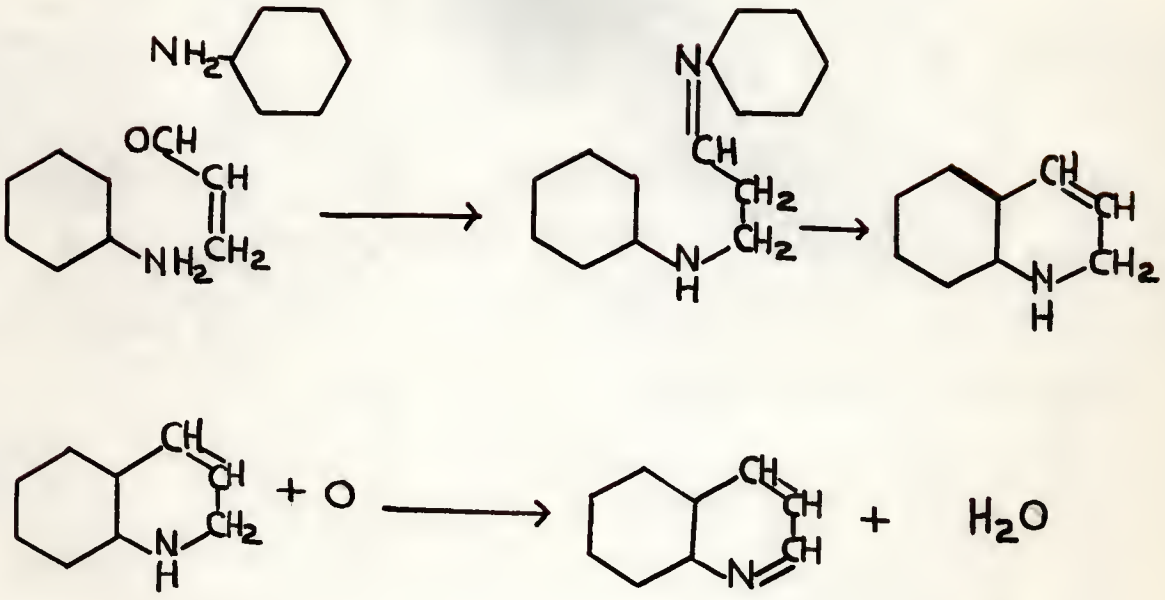
II. PREPARATION OF CARBON-14 LABELED 8-HYDROXYQUINOLINE

A. General Discussion

It was necessary to synthesize the carbon-14 labeled 8-hydroxyquinoline using the intermediates available. As stated, the carbon-14 was placed in two positions. In both cases a modified Skraup's synthesis (11) was used to yield the desired product. In the synthesis of 8-hydroxyquinoline-9-C^{14}, aniline-1-C^{14} (a product of Tracerlabs, Boston, Massachusetts) was reacted with glycerol and sulfuric acid in the presence of nitrobenzene and ferrous sulfate.

The Skraup reaction frequently takes place with great violence, and thus the ferrous sulfate is added as a moderator. In the case of the synthesis of quinoline, the reaction probably takes place through the following stages. The glycerol and sulphuric acid react together to form acrolein, CH_2=CH-CHO, which condenses with one molecule of aniline and combines additively with a second, thus yielding the anil of B-anilinopropionic aldehyde. Loss of a molecule of aniline and ring closure then take place, followed by oxidation of the resulting dihydroquinoline (12).

This scheme may be represented as follows:



It should be noted that in the preparation of the quinoline from aniline-1-C^{14} the observed specific activity of the resulting 8-hydroxyquinoline-9-C^{14} was quite low. This can be explained by assuming some of the reduced nitrobenzene reacted with the glycerol (or acrolein) to dilute the product. This point will be discussed later.

The sulfonation of quinoline was carried out according to the general method of E. Fischer (13). Using the procedure described, the major product was the 8-substituted sulfonic acid with a small contamination of 5-substituted product.

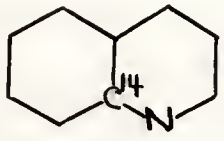
The 8-hydroxyquinoline-2,4-C^{14} prepared via labeled glycerol was of higher specific activity due possibly to the fact that any dilution effect of the reduced nitrobenzene would have no effect on the specific activity of the quinoline.

In these syntheses, using carbon-14, it was necessary to work in such a manner that none of the labeled compounds come in contact with the personnel or equipment in the laboratory. During this research the apparatus used was completely closed in glass systems or systems with adequate traps and good ventilation. It is unnecessary to use shielding with carbon-14, for the beta emission is very weak. It is unable to penetrate the walls of ordinary glass apparatus, and has a range of approximately 6 cm in air. The greatest hazard is not to the personnel but to the equipment and results of other workers. A small contamination could lead one to serious errors in mechanism studies.

B. Experimental

Individual details for the preparation of the carbon-14 labeled 8-hydroxyquinoline are given in this section. All temperatures are in degrees centigrade, and its symbol is omitted, conforming with present usage in scientific reports.

Synthesis of Quinoline-9-C^{14}



A one liter, round-bottomed flask was charged successively with powdered ferrous sulfate (6 grams), anhydrous glycerol* (50 ml, 0.60 moles), aniline-1-C^{14} (0.173 moles having a total activity of one millicurie or 5.76 microcuries/millimole, a Product of Tracerlab, Inc., Boston, Massachusetts), nitrobenzene (10 ml) and concentrated sulfuric acid (30 ml of 95%). The flask was swirled to mix the contents thoroughly and then attached to a water-cooled reflux condenser and heated over a small flame.

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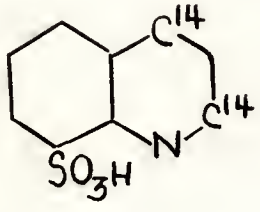
*Commercial glycerol is heated in a porcelain dish in the hood until the glycerol registers 180°.

Once homogeneity was obtained the flame was removed and the reaction allowed to proceed under self-induced reflux. After the initial reaction subsided, boiling was continued for four hours over a small flame.

The original flask was then connected for steam distillation and the unreacted nitrobenzene was removed. This portion was checked for radioactivity, but it showed only a very slight trace. After chilling the reaction mixture was made alkaline by the addition of sodium hydroxide (50 grams of sodium hydroxide dissolved in 150 ml of water). The mixture was steam distilled again and the pot residue was checked for radioactivity. It was found to have activity, thus some quinoline or other carbon-14 product remained. This portion was saved for the carbon-14 balance. The unreacted aniline was converted, through diazonium salt formation and decomposition, to phenol. The quinoline was extracted from the distillate with four 25 ml portions of benzene. The benzene was removed by distillation and the quinoline collected from 233-240° as a yellow liquid. The crude quinoline further purified by distillation yielded a pale yellow liquid (14.3 g) boiling at 236-239° at 763 mm. Inasmuch as the quinoline was an intermediate, its specific activity was not determined. The quinoline was dissolved in a mixture

of 50 ml of concentrated hydrochloric acid and 200 ml of water. After warming the clear solution, 30 grams of zinc chloride in 50 ml of 2 N hydrochloric acid were added. A quinoline-zinc chloride double salt crystallized as the solution cooled. The crystals were filtered, washed with 2 N hydrochloric acid, and decomposed with sodium hydroxide. Once more the quinoline was steam distilled, extracted with ether, dried in ether over potassium hydroxide and distilled after removal of ether. A water white preparation (10.5 grams) in 49 per cent yield and boiling at 238.5 to 239.5° at 758 mm was obtained (n_D^{20}=1.6238).

Synthesis of 8-Quinolinesulfonic-9-C^{14} Acid



Oleum (40 ml of 20 per cent) was charged into a 50 ml round bottom glass flask fitted with glass stirrer, mercury seal, thermometer and dropping funnel. Purified quinoline-9-C^{14} (10.5 grams, 77.5 millimoles obtained from the previous experiment) was dropped during twenty

minutes into the moderately stirred acid causing a rise in temperature to 160°. This temperature was maintained for four hours by application of external heat. After cooling to about 80°, the solution was poured into 50 ml of water and the sulfonic acid was allowed to crystallize for twenty-four hours at room temperature. Ice (25 grams) was added and after the ice melted, the sulfonic acid was filtered off and thoroughly sucked dry. Adhering sulfuric acid was removed by washing with ice water (three portions of 30 ml). The 8-quinolinesulfonic-9-C^{14} acid was dried on the steam bath. There was obtained 8 grams of light tan crystals. These were not assayed for radioactivity.

Alkaline Fusion of 8-Quinolinesulfonic-9-C^{14} Acid

Sodium hydroxide (7 grams) and potassium hydroxide (7 grams) were charged with one ml of water in a thick-walled, open porcelain dish of 250 ml volume, fitted with a sturdy stirrer and a thermometer inserted in the dish. The dish was heated with a Bunsen burner. After the alkali had become soft, stirring was started and the temperature was raised to 245°. Finely powdered 8-quinolinesulfonic-9-C^{14} acid (8.0 grams) was added during ten minutes to the melt to maintain a temperature of 240-250°. The temperature was not allowed to rise above 250°, for low yields resulted at higher temperatures. The fusion process is exothermic and the temperature can be controlled somewhat by the

addition of the 8-quinolinesulfonic-9-C^{14} acid. After the addition of the sulfonic acid, the melt was stirred ten minutes longer at 240°. The melt was then poured on a metal sheet, allowed to cool, broken up into small pieces and transferred to a 150 ml round bottom glass flask fitted with stirrer, mercury seal, dropping funnel and reflux condenser. Water (40 ml), part of which was used to clean the porcelain dish, was added; the stirrer put into operation and sulfuric acid (95 per cent) dropped in until a pH of 7.0 was reached. Although the temperature rose during this process to the boiling point, it was advisable to reflux thoroughly before testing to make certain that all alkaline particles were dissolved. The final pH of 7.5 was determined accurately by means of a pH meter. After cooling to about 50°, the stirrer was stopped and the contents of the flask were allowed to remain at room temperature for about ten hours. The solid, consisting of 8-hydroxyquinoline-9-C^{14}, alkali sulfate, and a black tar was filtered, washed twice with ten ml of water, and dried superficially by air. The 8-hydroxyquinoline-9-C^{14} was steam distilled in 250 ml of water and purified by several recrystallizations from aqueous ethanol. There was obtained 4.46 grams (55 per cent of theory) of the compound with a specific activity of 4.37 microcuries/millimole and melting at 75-76°. The mixed melting point

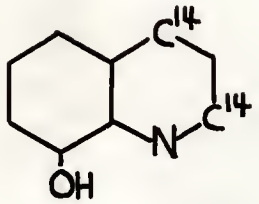
with an authentic sample was 75.5-76°. The overall yield based on aniline-1-C^{14} was 17.8 per cent.

The radioactive balance is as follows:

	Specific activity in μc/mM*	Total activity in microcuries
Initial reactant (Aniline-1-C^{14})	5.76 μc/mM	1,000 μc
Products (8-hydroxyquinoline-9-C^{14})	4.73 μc/mM	136 μc
Unreacted Aniline-1-C^{14} converted to phenol		122 μc
Pot residue from first steam distillation		202 μc
Quinoline distillation waste		311 μc
Quinoline-hydrochloric acid		97 μc
Final steam distillation residue		57 μc
	Total	925 μc

Per cent accounted for (92.5%)

Synthesis of 8-Hydroxyquinoline-2,4-C^{14}



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* c/mM expresses microcuries per millimole

A pear-shaped 100 ml flask was fitted with a condenser, thermometer, and stirrer, o-nitrophenol (0.5 grams), o-aminophenol (1.0 gram, 9.18 millimoles) and glycerol-1, 3-C^{14}*, (432 microcuries, 10.8 millimoles) were added and mixed with a little heating. When the mixture became homogenous, the condenser was placed on a flask and concentrated sulfuric acid (3 ml) was added through the condenser. After the initial reaction slowed down, the mixture was refluxed gently for five hours. At this point the flask was attached to a steam distillation apparatus and the excess o-nitrophenol was removed from the acid solution. Sodium hydroxide was added followed by enough sodium carbonate to adjust the pH to a value of 7.5. The mixture was again steam distilled to remove the 8-hydroxyquinoline-2,4-C^{14}. The 8-hydroxyquinoline-2,4-C^{14} distilled slowly, and 150 ml of water were required to remove all the product. The resulting material was recrystallized several times from aqueous ethanol. There was obtained 0.55 grams (41 per cent of theory) of product with a melting point of 75-76° and a specific activity of 46.6 microcuries/millimole.

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*A product of Isotopes Specialties, Pasadena, Calif.

The radioactive balance is as follows:

	Total Activity
Initial reactants - (glycerol-1,3-C^{14})	432 μc
Product - (8-hydroxyquinoline-2,4-C^{14})	177 μc
Residue from steam distillation	241 μc
Total	418 μc

Per cent accounted for (97%)

III. RADIOACTIVE MEASUREMENTS

A. General Discussion

Ruben and Kamen (13) were able to isolate and identify carbon-14 from a graphite probe which had been bombarded with deuterons of low energy (3-4 Mev). This offered very small amounts of material for study. The current production by the reaction $N^{14}(n,p)C^{14}$ as suggested by Reid and Urey (14) is by irradiation of saturated ammonium nitrate solutions in the Clinton pile (15). The half-life of carbon-14 based on weighted averages is 5,568 years (16). Carbon-14 emits beta particles with a maximum energy of 0.155 ± 0.002 Mev. (17) The approximate range of the beta particle in air is 6 cm. The beta particles are unable to penetrate the walls of ordinary glass apparatus. The dangers associated with the use of carbon_14 are small with respect to personal health hazards if care is taken to prevent outside contamination of the glass apparatus used. There are some indications that carbon-14 can accumulate in certain hard tissues of the body but this has not been established as fact (18).

The apparatus in which the experiments using carbon-14 are carried out can be easily cleaned by washing with water and soaking in hot chromic acid solution. It is suggested that detergents are not suitable unless the acid bath treatment is also used. The detergent particles tend to cling to the glass surface.

For most reactions involving carbon-14, ordinary apparatus or slightly modified apparatus was used since the level of beta activity was not extremely high. Many compounds used for the preparation of intermediates in tracer work are prepared using vacuum systems (19).

Three types of measurements were used in this work, Either solids, liquids or gases were used for counting depending on the applicability and efficiency demanded by the particular experiment. The solid samples were of two kinds, pure chemical material and barium carbonate derived from combustion of the compound to carbon dioxide, and its subsequent precipitation as barium carbonate. The combustion was carried out by Van Slyke-Folch method (20) with several modifications described in the experimental section in full detail. The carbon dioxide was then precipitated as $BaCO_3$ and

counted as such. The pure chemical materials were counted by plating on aluminum dishes in very thin layers. 8-Hydroxyquinoline-9-C^{14} was counted by placing a known amount in a dish and adding just enough copper to form the 2 to 1 copper chelate. It is essential that the samples used be nonvolatile and the correct self-adsorption adjustments be made.

The liquid samples were counted according to the general method of Schwebel, Isbell and Karabinos (21). The major modification was the use of solvent. They used pure formamide with less than 10 per cent water while various solutions of formamide and phosphoric acid and water were used in this work. The liquid method of counting is superior to the direct solid measurement where variations in self-adsorption, back-scattering and fore-scattering, and (in counting on plastic backings) by the presence of static charges that distort the field. These complications are not noted in liquid counting because the environment of the solute is strictly reproducible and solutions have little tendency to hold static charges. The drawback to liquid counting lies in the limited number of solvents that can be used and the low efficiency (1%) as compared to thin solid samples (50%).

The gaseous measurements were made by first converting the carbon-14 source to carbon dioxide which was

trapped and stored as sodium carbonate. The carbon dioxide was liberated and allowed to enter an ionization chamber. The activity of the carbon dioxide thus produced was measured on a Cary Model 30 Vibrating Reed Electrometer (a product of Applied Physics Laboratory, Pasadena, Calif.). The radioactivity determination by this method is simpler, faster, more accurate and less expensive than other methods (22, 23, 24, 25, 26). It is possible to measure as little as 10^{-12} curies of radioactivity using the vibrating reed (26). The difficulties associated with the vibrating reed electrometer are almost entirely constructional. However, inasmuch as they are almost always purchased, these are reflected only in the cost. In the vibrating reed electrometer, the D.C. input signal from the ionization chamber filled with radioactive carbon dioxide is converted to A.C. by using mechanical energy to move the impressed charge through an electrostatic field. The A.C. signal thus produced, which is a measure of the impressed charge, is amplified in a stable A.C. amplifier and then rectified and used to drive the recording meter. A portion of the rectified A.C. is applied as negative feedback to give very high stability (27).

B. Experimental

Solid Counting Methods

Individual details not included in the general discussion are given in this section. Several procedures which have been found useful in assay of radioactive carbon include: 1) a wet oxidation method for the determination of total carbon and of the specific activity of carbon in organic materials, and 2) a method for collecting barium carbonate samples on filter paper disks for counting.

The combustion fluid of Van Slyke and Folch (20) and the various reagents were prepared as follows:

Combustion Fluid.

A 500 ml Pyrex Erlenmeyer flask with a glass stopper is charged with chromic anhydride (12.5 g), followed by syrupy phosphoric acid (84 ml of specific gravity 1.7), and fuming sulfuric acid (167 ml of acid with 20 per cent excess SO_3). Heat was applied with occasional gentle agitation and with the stopper off until the temperature reaches 150°. The flask is cooled while covered with an inverted beaker. When cool, the stopper is inserted and the inverted beaker is retained as added protection from dust. This reagent loses

strength on standing. It was tested for CrO_3 content at two-week intervals as follows: The oxidation reagent (2,5 ml) was diluted to 25 ml. Three milliliters of the dilution were placed in a 50 ml Erlenmeyer flask and water (5 ml) and KI solution (10 ml of a 10 per cent solution) were added, and the flask was allowed to stand for five minutes. The free iodine was then titrated with 0.1 N $Na_2S_2O_3$. The number of ml of $Na_2S_2O_3$ required divided by 9 gives molar concentration of CrO_3 in the combustion fluid. The CrO_3 concentration should be at least 0.475 N. More CrO_3 may be added to make up any deficiency observed. Upon addition of the extra CrO_3, the solution may be reheated to 150°.

Potassium Iodate.

Reagent grade KIO_3 was powdered in a mortar and stored in a glass stoppered bottle. It was observed on one occasion that after a month or more in the bottle, the KIO_3 failed to dissolve readily in the combustion fluid. This was corrected by substituting freshly powdered KIO_3.

Sodium Hydroxide - Hydrazine Solution.

A liter of approximately 0.8 N NaOH is made from carbonate-free saturated NaOH (a saturated solution of NaOH in a paraffin-lined bottle was allowed to stand several weeks until the insoluble carbonate settles out)

and freshly boiled distilled water. The 0.8N NaOH is prepared and stored in an aspirator bottle protected from atmospheric CO_2 by a soda-lime absorber. Two grams of hydrazine sulfate were placed in a second aspirator bottle, calibrated at 100 ml and protected by a soda-lime tube. The air above the hydrazine sulfate is replaced with CO_2-free gas, and the 0.8N NaOH is drawn in from the stock bottle to the 100 ml mark. The hydrazine sulfate is dissolved by agitation, and the resulting reagent is drawn into a reservoir burette which has been flushed out with CO_2-free gas. The openings of the burette are protected with soda-lime tubes. This solution loses hydrazine strength on standing, and should be made up fresh once a month.

Barium Hydroxide - Barium Chloride Solution.

A small portion of a saturated solution of $Ba(OH)_2$ is standardized against standard HCl. One liter of 0.3N $Ba(OH)_2$ was then made by appropriate dilution of a portion of the saturated solution. To 800 ml of the 0.3N $Ba(OH)_2$ was added a $BaCl_2$ solution (160 ml containing 12 g of $BaCl_2 \cdot 2H_2O$ per 100 ml). These volumes were measured in a graduated cylinder. The final solution was drawn by suction into a bottle previously flushed with nitrogen gas. The solution was drawn into the burette

through a sintered glass filter funnel of "M" porosity to remove any $BaCO_3$ particles. All openings were protected with soda-lime tubes.

Methyl Orange Indicator.

A 1 per cent solution of methyl orange in water.

Phenolphthalein Indicator.

A 0.5 per cent solution of phenolphthalein in 95 per cent ethanol.

Standard 0.1N Hydrochloric Acid.

An approximately 0.1N HCl solution was accurately standardized.

Procedure:

A modification of the method of Van Slyke and Folch (20), as described by them under the heading "micro and submicro combustions" was used for the wet oxidations. In the original procedure, the CO_2 formed is pumped into the Van Slyke chamber and trapped in alkali by repeated alterations of pressure. In the method described here, the last portions of CO_2 are frozen out in the receiver flask while the generator is being strongly heated.

The sample to be oxidized, which should contain from 2 to 2.5 mg carbon, and which should be fairly dry is placed at the bottom of the combustion tube of the apparatus pictured in Figure 1. The joint of a receiver flask is lubricated with stopcock grease and 2 ml of the

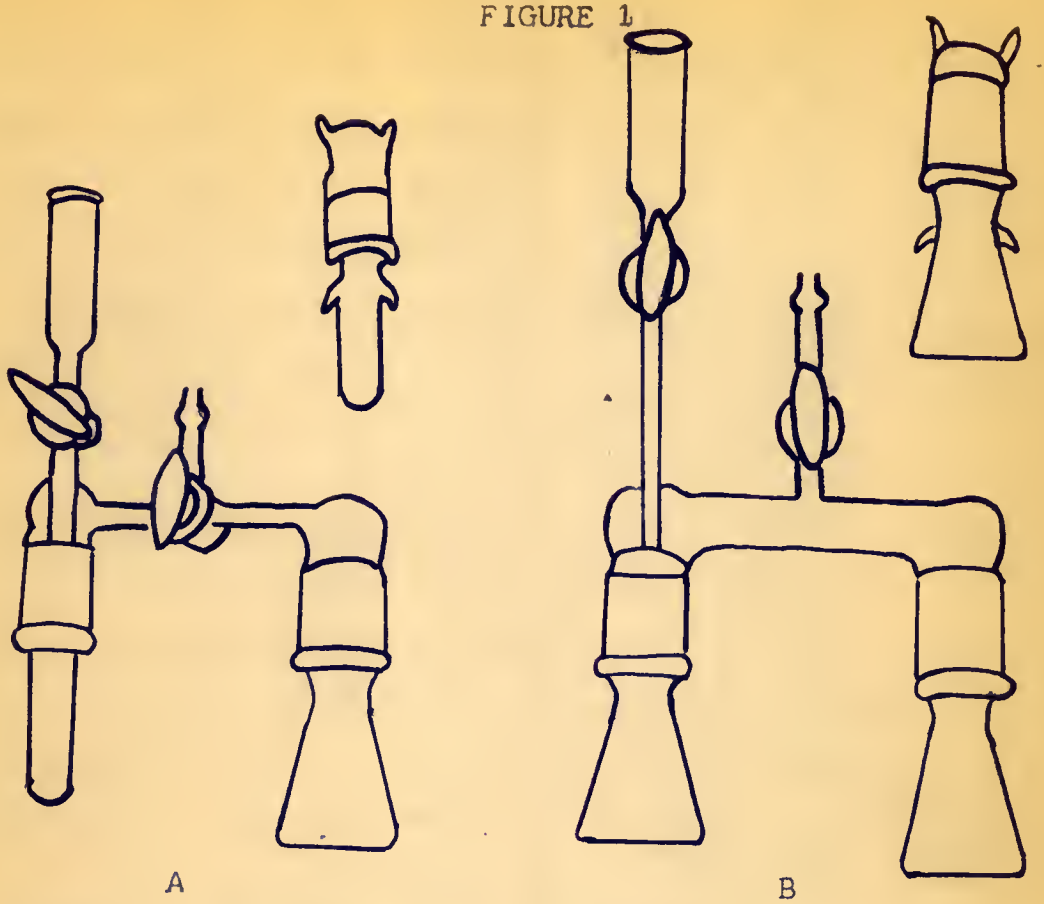
NaOH-hydrazine solution are measured into the flask, using a stream of nitrogen or CO_2-free air to flush the flask before and during the addition of the alkali. The prepared receiver flask is capped and set aside.

Powdered KIO_3 (200 mg) was added in a small glass dish. The dish (30 x 5 x 5 mm) serves as a boiling chip during the later heating period. The stopcock of the acid dropper and the glass joint of the combustion are lubricated with syrupy H_3PO_4. The apparatus is assembled except for the receiver flask.

With the water pump in operation and connected to the open central stopcock, the receiver flask, previously filled with alkali, was put in place. Both sides of the apparatus are evacuated to the limit of a water pump. The center stopcock was then turned so the two arms are open to each other but closed to the atmosphere. The water pump hose is disconnected and the apparatus supported in such fashion that the receiver flask can be immersed later in liquid air. Now 2 ml of the combustion fluid is allowed to run into the tube containing the sample. The acid cup should be marked at the 2 ml level to aid in this addition.

A minute or two of heating with a small flame brings the combustion liquid to boiling, and this

FIGURE 1



APPARATUS USED IN WET COMBUSTION AND CARBON DIOXIDE DETERMINATION. Scale: 1mm represents 2.5mm in original.

A-Bottom, assembled combustion apparatus; top, combustion tube with cap. All joints are ₹ 24/40.

B-Bottom, assembled apparatus for CO_2 collection in $Ba(OH)_2$-$BaCl_2$; top, flask with cap. All joints are ₹ 24/40.

boiling is continued for one to two minutes with some agitation of the receiver flask. The receiver flask is then dipped nearly to the glass joint in liquid air or a dry ice-acetone mixture and gentle boiling of the oxidation fluid is continued for three minutes. Finally, the oxidation mixture is momentarily boiled vigorously, so that it occupies most of the volume of the oxidation tube, and the T-bore stopcock is quickly turned so that the oxidation tube is opened to the water pump while the receiver flask remains closed. The receiver flask is allowed to warm to room temperature and finally heated with warm water and agitated slighly to insure complete absorption of CO_2. Nitrogen gas was allowed to run into the receiver flask to bring the pressure to atmospheric, it was capped and set aside for either precipitation as $BaCO_3$ or liberation of the CO_2 for measurement with the vibrating reed electrometer. The combustion tube is rinsed with distilled water and set aside to dry. The rest of the apparatus, unless badly contaminated with iodine, can be used over again without cleaning.

It is possible by a modification of the method of Van Slyke, Mac Fayden, and Hamilton (28) to determine quantitatively the carbon content of the sample. Samples of pure benzoic acid gave figures for carbon, by this

acidimetric determination of the total carbon dioxide produced of 99.4 to 100.3 per cent of the theoretical values. The original procedure involved the transfer *in vacuo* of CO_2, split by ninhydrin from alpha-amino acids, to $Ba(OH)_2$-$BaCl_2$ solution. The present method described provides for introducing acid into a carbonate solution in the evacuated system. The transfer of CO_2 *in vacuo* to $Ba(OH)_2$-$BaCl_2$ solution is done in the same way as in the original procedure. This transfer of CO_2 from the NaOH-hydrazine solution serves two purposes: 1) acidimetric determination of the total carbon dioxide produced in the wet oxidation step is possible if desired, and 2) a pure $BaCO_3$ precipitate is obtained for counting purposes. Impure $BaCO_3$ often results from precipitation of carbonate-hydroxide solutions with $BaCl_2$ (29,30). This may be guarded against by addition of NH_4Cl prior to the $BaCl_2$ (31). Very slight co-precipitation of acid or alkaline salts occurs in the absorption of gaseous CO_2 by the Van Slyke $Ba(OH)_2$-$BaCl_2$ mixture, as evidenced by the quantitative results in the back-titration with acid.

Apparatus (b) in Figure 1 was used for the formation of the $BaCO_3$ samples. First 2.5 ml of the $Ba(OH)_2$-$BaCl_2$ solution was measured into a receiver flask under

a flow of nitrogen gas. A small amount of grease is used, for particles of grease in the $BaCO_3$ interfere with filtration onto the counting papers. Second, the stopcock of the acid dropper is lubricated with syrupy phosphoric acid and the NaOH-hydrazine solution and the $Ba(OH)_2$-$BaCl_2$ solution was set in place on apparatus (b). The water pump was put in operation and both arms were evacuated while the NaOH-hydrazine solution was immersed in liquid air or dry ice-acetone mixture.

Approximately 1N H_2SO_4, with one drop of methyl orange, was allowed to drop into the NaOH-hydrazine solution until the methyl orange just maintained its acid color. The acid side was then immersed in boiling water with agitation. After two minutes the $Ba(OH)_2$-$BaCl_2$ side was immersed in liquid air or dry ice-acetone mixture for three minutes. The apparatus is then allowed to stand at room temperature for five minutes or until the ice melts in the $Ba(OH)_2$-$BaCl_2$ side. This sample may then be filtered for counting or titrated with standard HCl.

A sample of the $Ba(OH)_2$-$BaCl_2$ solution, measured out as previously described to exclude atmospheric CO_2, was titrated directly with the standard HCl. The difference between this titration and the titration of the unknown measures the total $BaCO_3$ through which the

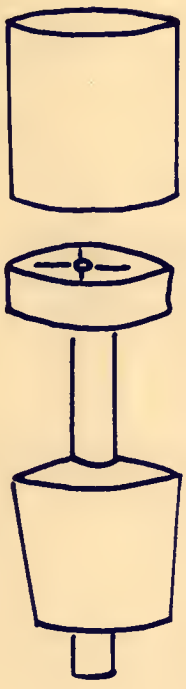
radioactive carbon is distributed. A blank run must be carried through all steps if an exact carbon determination is desired. It may be necessary to dilute the NaOH-hydrazine solution with "cold" Na_2CO_3 if the original material is of high specific activity.

The collection of $BaCO_3$ and counting of the sample is of great importance and thus the procedure will be fully covered. A standard solution of carbon-14 as $Na_2C^{14}O_3$ can be prepared from the $BaC^{14}O_3$ as it is shipped from the Isotopes Division, Atomic Energy Commission. The specific activity and the isotope ratio are specified for each shipment. One such shipment is selected to serve as a standard for comparison and is diluted with enough C^{12} so a suitable counting rate is obtained with a 20-30 mg sample of $BaCO_3$. The most convenient method is to obtain a standard $Na_2C^{14}O_3$ sample from the National Bureau of Standards and a $BaCO_3$ sample is prepared as in the method described below. The method of collection and counting the $BaCO_3$ samples is a slight modification of a method demonstrated by A. Schwebel at the National Bureau of Standards. The differences are in the fact that the quantitative transfer of $BaCO_3$ to the paper is not attempted, since the total amount of $BaCO_3$ containing the activity to be measured is known when

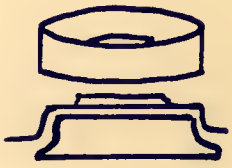
precipitates are prepared by the titration method described above. Figure 1 and 2 shows the apparatus used. The stainless steel filter apparatus (a product of Tracerlab, Inc., Boston, Mass.) was used in combination with brass mountings. This filter allows the paper used for collection of the precipitate to be performed and weighed before the $BaCO_3$ is deposited. The surface area of the paper disk thus formed is 2.895 cm^2. A quantity of $BaCO_3$ suspension containing 20 to 30 mg $BaCO_3$ is poured in the filter with the water pump on. The precipitate is washed at least three times with 10 ml portions of distilled water and finally with two 10 ml portions of ethanol. The paper is then removed to an oven at 110° to dry for one hour. The weight of dried $BaCO_3$ is obtained by comparing the total weight of the precipitate plus the paper and the original paper weight. This gives the fraction of the original total $BaCO_3$ which appears on the paper, and also allows a calculation of the thickness of the $BaCO_3$ layer, expressed in milligrams per square centimeter. The filtering surface area of the various disks are of equal magnitude. Using measurements of the disk diameters, the thickness of the $BaCO_3$ samples in mg per sq cm are calculated, and after

FIGURE 2

$BaCO_3$ FILTRATION APPARATUS



STAINLESS STEEL
FUNNEL



BRASS DISK AND RING

The brass disk is used to mount the $BaCO_3$ after filtration on preformed paper in the stainless steel funnel.

correcting for background they are translated to counting rates at "infinite thickness" by means of the $BaCO_3$ self-absorption curve of Reid et al (32).

In calculating the specific activity of a $BaCO_3$ precipitate counted in the manner described, the number of counts per minute observed were first corrected for the background count, next translated to "infinite thickness" as described above, then divided by the paper disk area in sq cm, and finally compared with the number of counts per minute per sq cm of paper surface given by an "infinite thickness" of standard $BaC^{14}O_3$.

The carbon-14 standard used was NBS #1* with an activity of 1280 dps/ml of solution. This sodium carbonate solution is the same one used to calibrate the vibrating reed electrometer.

A second method of solid counting involves direct plating, of the material either pure or in some biological substrate, on aluminum dishes. This method does not allow as quantitative results as the $BaCO_3$ method, but by proper calibration curves to correct for the self-absorption of the substrate, this method has proved to be satisfactory for quantitative determinations of biological materials.

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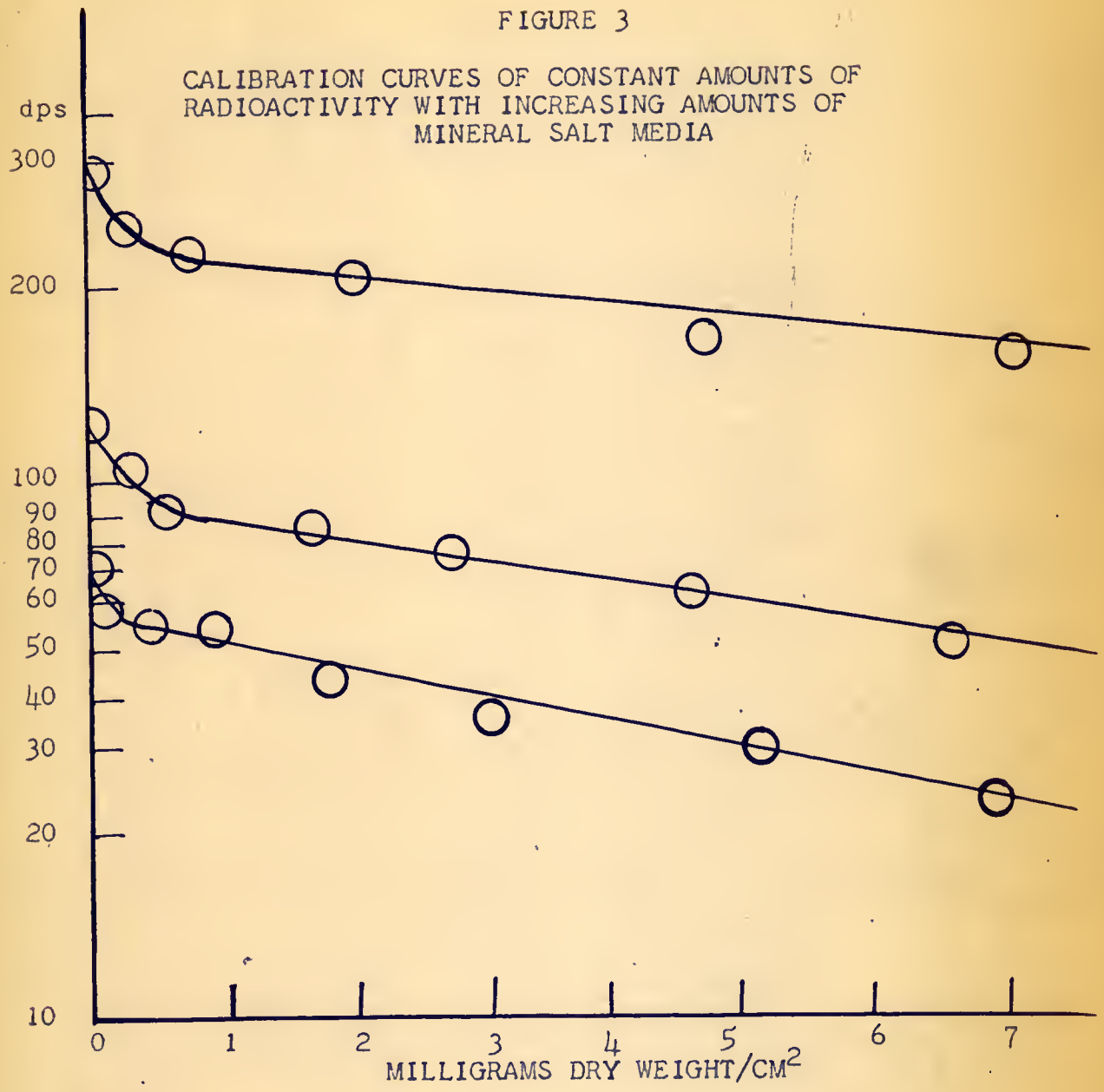
*NBS #1 is a calibrated Na_2CO_3 standard from the National Bureau of Standards.

In preparing calibration curves, increasing amounts of non-radioactive material were added to a series of tubes, each containing a known constant amount of an aqueous solution of 8-hydroxyquinoline-9-C^{14} . The contents of tube were throughly mixed and transferred to aluminum dishes, dried with an infrared lamp and a hot air blower on a horizontal turntable. The specific activity of the 8-hydroxyquinoline-9-C^{14} was known so aqueous solutions were plated with various amounts of substrate. The data plotted on semi-log scale in Figure 3, indicate that the addition of very small amounts of substrate to a constant amount of radioactivity produces an initial decrease in measured activity. This drop amounts to about 20 per cent of the original activity determined by plating and counting an aqueous solution of 8-hydroxyquinoline-9-C^{14} to which no substrate has been added. Additional plates made by the above method, using increasing amounts of substrate, show a constant decrease in measured activity consistent with the anticipated absorption curve for weak beta particles. By using such calibration curves and interpolating back to the ordinate, better than 90 per cent of the original activity in a system of _in vitro_ studies with fungi was accounted for.

FIGURE 3

CALIBRATION CURVES OF CONSTANT AMOUNTS OF RADIOACTIVITY WITH INCREASING AMOUNTS OF MINERAL SALT MEDIA



Liquid Counting Method

The usual method of analysis for carbon-14 involves oxidation of the organic substance and the collection and precipitation of the CO_2 by barium hydroxide to form $BaCO_3$. In some cases this method is the only recourse, but it is rather long and difficulties are encountered in obtaining a uniform sample. A modification of the method developed by Schwebel, Isbell, and Karabinos was used in most of this work (21). The procedure consists of making a solution of the radioactive material in one milliliter of a suitable solvent. A suitable solvent would be non-volatile and should have a relatively high surface tension. This solution is pipetted into a circular 1 ml cell with an inside diameter of 37 mm and a depth of 1.1 mm. The cell was constructed of stainless steel. The cell is used in a gas-flow proportional counter, Nuclear Measurements Corporation - Model PC-1. The only modification needed was a cooling system to keep the cell and the sample cool. By cooling the cell it has been found that up to 10 per cent water can be tolerated without a detectable change in reproducibility or accuracy. Since the depth of the liquid for counting purposes is essentially of

"infinite thickness", the counts are proportional to the carbon-14 concentration of the liquid. It was possible to calibrate the PC-1 proportional counter and determine its efficiency using a standard solution of D-mannitol-1-C-14 supplied by the National Bureau of Standards. This method is not applicable if the activity is very low since only about one per cent of the total activity can be determined. It is also important to determine the effect on efficiency caused by the solvent used in the analysis. For example, the activity of a sample of 8-hydroxyquinoline-9-C^{14} was 790 dps/mg in formamide, 505 dps/mg in 90 per cent phosphoric acid, and 600 dps/mg in an equal mixture of the two solvents. If a solvent couple such as formamide-phosphoric acid is to be used, it is possible to determine the efficiencies at several concentrations and plot concentration vs. activities. From this graph the intermediate efficiencies can be determined at any concentration in a manner similar to that for direct plating. In the analysis of solutions of 8-hydroxyquinoline-9-C^{14}, a number of problems arise. The material counted must be less volatile than the solvent. 8-Hydroxyquinoline is volatile enough to contaminate the counter when counted in formamide

solution. For this reason it was necessary to place 0.25 ml of phosphoric acid in each 10 ml sample of medium taken for analysis. This allowed evaporation of the water without loss of 8-hydroxyquinoline, now chemically combined as a non-volatile phosphate. It was necessary to prepare a control in a medium similar to the one used in the experiment. The 10 ml sample taken for analysis was evaporated by blowing a warm air stream over the solution. When about 0.30 ml remained, the test tubes were dried further over P_2O_5 in a vacuum. When the volume was 0.25 ml, 1.75 ml of formamide was added. a one ml volume was placed in the cell and counted. Generally 5,000 counts were recorded which represents a one per cent probable error.

The method of analysis was worked out using several combinations of formamide-phosphoric acid. In Table I the count of solutions of 8-hydroxyquinoline-9-C^{14} in growing cultures of Aspergillus niger v. Tiegh* at various pH was made in 95 per cent phosphoric acid.

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* The Aspergillus niger v. Tiegh culture used in this work was supplied by the Pioneering Research Laboratories U.S. Army Quartermaster Corps., Philadelphia, Penn.

TABLE I

Analysis of

8-hydroxyquinoline-9-C^{14} in 95% H_3PO_4

(Total Volume 1.0 ml H_3PO_4)

Control (10-3M 8-hydroxyquinoline-9-C^{14})	pH 3	pH 5	pH 7
71.6 dps/ml	68.5 dps/ml	69 dps/ml	67.5 dps/ml

This method involved pipetting 95 per cent phosphoric acid, a procedure which was extremely difficult. In order to facilitate pipetting, 0.75 ml of formamide was added instead of phosphoric acid and the results in Table II were obtained.

TABLE II

Analysis of

8-hydroxyquinoline-9-C^{14} in 25% H_3PO_4

(Total Volume - 0.25 ml 95% H_3PO_4 and 0.75 ml Formamide)

Control (10-3M 8-hydroxyquinoline-9-C^{14})	pH 3	pH 5	pH 7
95.8 dps/ml	89.8 dps/ml	90.8 dps/ml	88.8 dps/ml

The most critical part of this analysis is proper dilution and pipetting so the solutions are of the correct concentration. The mixture of 0.75 ml of formamide and 0.25 ml of phosphoric acid was still difficult to pipette. Special test tubes were constructed with a 1 ml bulb at the bottom. This was done so that after evaporation of the water from the sample, the 0.25 ml of phosphoric acid would be in this bulb. Enough formamide was added to give 1 ml volume. One more ml of formamide was added and the contents were well mixed. One ml was withdrawn and placed in the cell. This method produced a fluid easy to pipette. The actual efficiency of the counting was improved by this method. The following results obtained are shown in Table III.

TABLE III.

Analysis of

8-hydroxyquinoline-9-C^{14} in 14% H_3PO_4

(Total Volume - 0.25 ml H_3PO_4 and 1.75 ml of Formamide)

Control	pH 3	pH 4	pH 5	pH 7
110 dps/ml	107 dps/ml	106.8 dps/ml	108 dps/ml	101.2 dps/ml

Gaseous Counting Method

The procedure in this method is identical in the first stages to the $BaCO_3$ method. The $Na_2C^{14}O_3$ solution

as prepared in the solid method is placed in a vacuum line as shown by Calvin (19) and acidified with H_2SO_4. The $C^{14}O_2$ is then placed in the evacuated ionization chamber. Cold CO_2 is then flushed into the system by alternating freezing and thawing of the incoming CO_2. Enough CO_2 is added to bring the ionization chamber to a standard pressure, 760 mm for this work. The chamber is closed, removed from the vacuum line ready for counting. The chamber is connected to the vibrating reed electrometer and the rate of voltage drift in millivolts per second is determined. A blank determination of the chambers with "cold" CO_2 only is run to evaluate the background drift rate. The ionization chambers were calibrated using NBS # 1 standard. Using various dilutions of the $Na_2C^{14}O_3$ the drift rate per second was calibrated in terms of disintegrations per second. A drift rate of 0.1543 millivolts per second is equal to one disintegration per second. It is possible to convert the ion current to disintegrations directly if the capacitance of the electrometer is accurately known. However, this is not necessary when good $Na_2C^{14}O_3$ standards are available as was the case in this work.

Instruments Used.

An alpha-beta-gamma Porportional Counter, Model PC-1 (Nuclear Measurements Corp., Indianapolis, Indiana,) was used for the solid and liquid counting. This is a "P-gas" (90 per cent Argon, 10 per cent methane) windowless type flow counter. For point sources the geometry is 50 per cent of a solid angle. The other instrument used was a Cary Model 30 Vibrating reed electrometer (Applied Physics Corp., Pasadena, Calif.) with a Brown Electronic Recorder. The Model 30 was equipped with a No. 3097 turret switch with resistors of 10^{12}, 10^{10} and 10^{8} ohms. For the work reported here, the resistors were not used.

IV. STUDIES ON 8-HYDROXYQUINOLINE

A. Mycelial* Uptake

The first condition in determining the manner in which a fungicide is operating involves a determination of the actual amounts of the toxicant taken into the mycelium or spores of a fungus. In an effort to obtain this information, several series of experiments were conducted at various hydrogen ion concentration and at two concentrations of 8-hydroxyquinoline.

The toxicity of 8-hydroxyquinoline toward A. niger at various hydrogen ion concentrations increases as the pH approaches 7. With this in mind the determination of the amounts of 8-hydroxyquinoline actually taken up by the fungus at various pH values was determined first.

A salt medium of the following composition was used:

0.5 g $MgSO_4$ 1.0 g KH_2PO_4 0.5 g KCl 3.0 g KNO_3
20.0 g Dextrose 1000 ml water

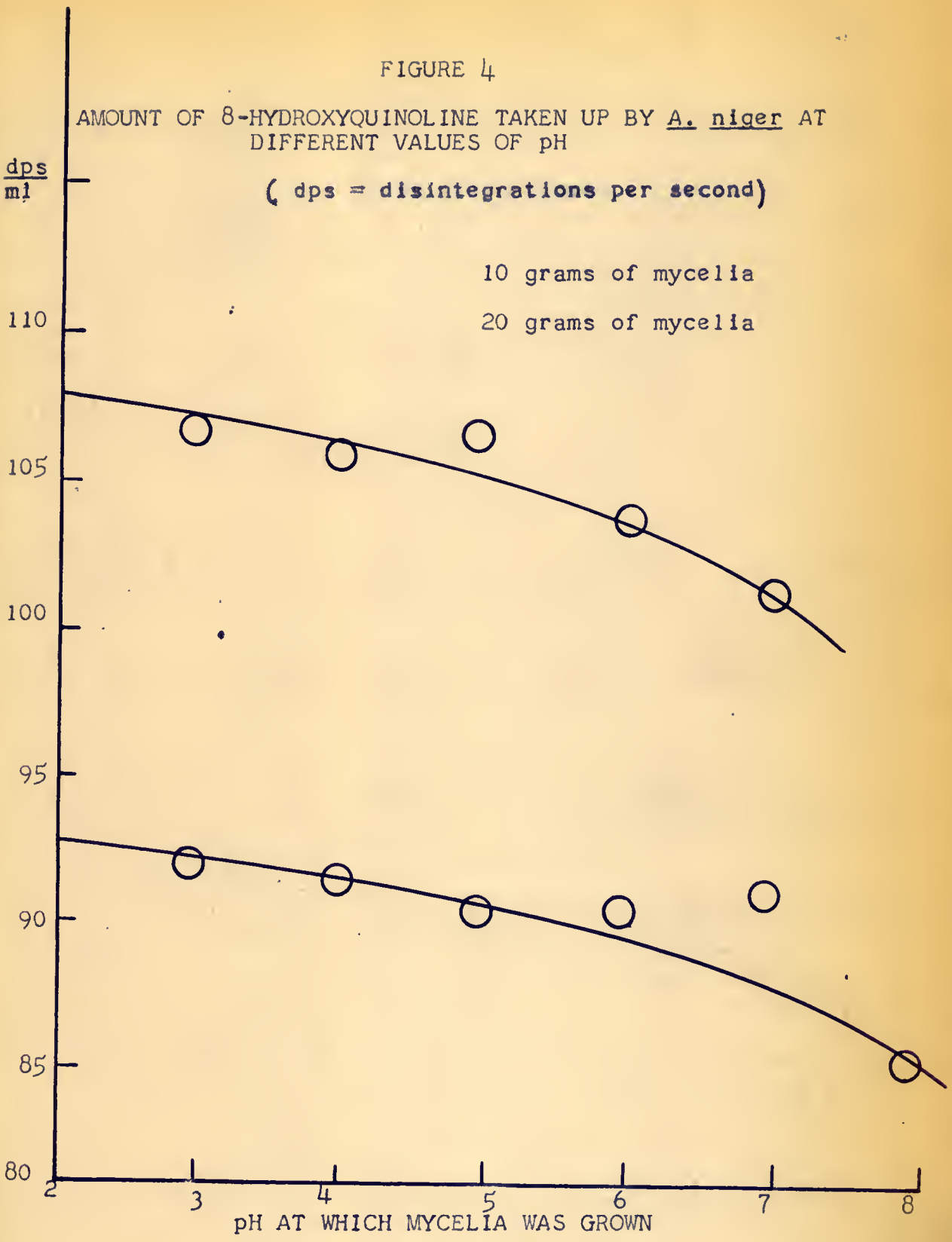
This medium was selected for its suitability in allowing macimum growth of A. niger under controlled conditions. Several liquid cultures were inoculated with a suspension of pre-germinated spores and the volume was

*Mycelia as used throughout should be considered as the mycelium of the fungus Aspergillus niger.

carefully measured in order to maintain uniformity in cultures. The cultures were shaken at regular intervals to aerate the medium and allow the fungus to grow below the surface. After 48 hours growth, the cultures were adjusted to various pH values, using buffer solutions to yield the correct hydrogen ion concentration. One ml of 0.1 M 8-hydroxyquinoline-9-C^{14} was added to each flask and to the non-inoculated control. This gave a solution of 10^{-3} M or slightly more due to evaporation. The evaporation, however, was not critical inasmuch as the control was treated in a similar manner. A 10^{-3}M solution of 8-hydroxyquinoline counted in phosphoric acid showed a count of 71.2 dps/ml while the control gave 70.6 dps/ml. This is good agreement. The cultures were again allowed to stand with periodic shaking for 24 hours. At this time 30 ml of the flask contents were centrifuged and 10 ml of the clear mycelia-free liquid were pipetted into a test tube for analysis as described under the liquid counting method. Figure 4 shows that the uptake of 10^{-3} M 8-hydroxyquinoline, using two different weights of mucelia, increases with increased pH. The same series run with 20 g of mycelia in 100 ml of solution was repeated using mycelia that had

FIGURE 4

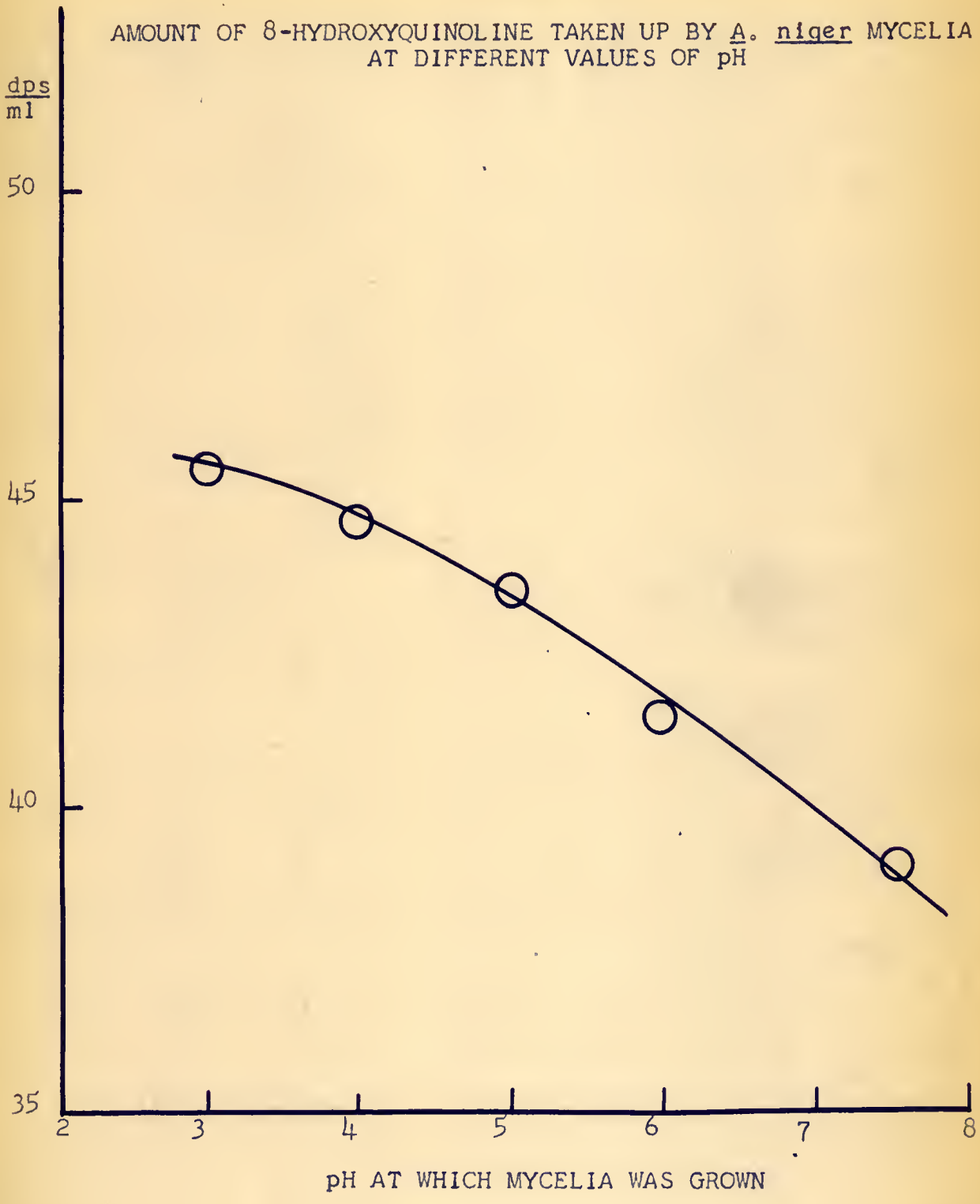
AMOUNT OF 8-HYDROXYQUINOLINE TAKEN UP BY A. niger AT DIFFERENT VALUES OF pH



of solution was repeated using mycelia that had been ground in a Waring blendor. Similar results were obtained. This indicates that the pH effect does not necessarily control the ability of the neutral ion to enter the fungus mycelia but that it could be due to a difference in reactivity of either the fungal material or the 8-hydroxyquinoline. The specific activity (1,118 dps/mg) of 8-hydroxyquinoline-9-C^{14} was not high enough to go to lower concentrations so the 8-hydroxyquinoline-2,4-C^{14} prepared from glycerol-1,3-C^{14} having a specific activity of 5,110 dps/mg was used in a series of studies run at concentrations of 10^{-4}M.

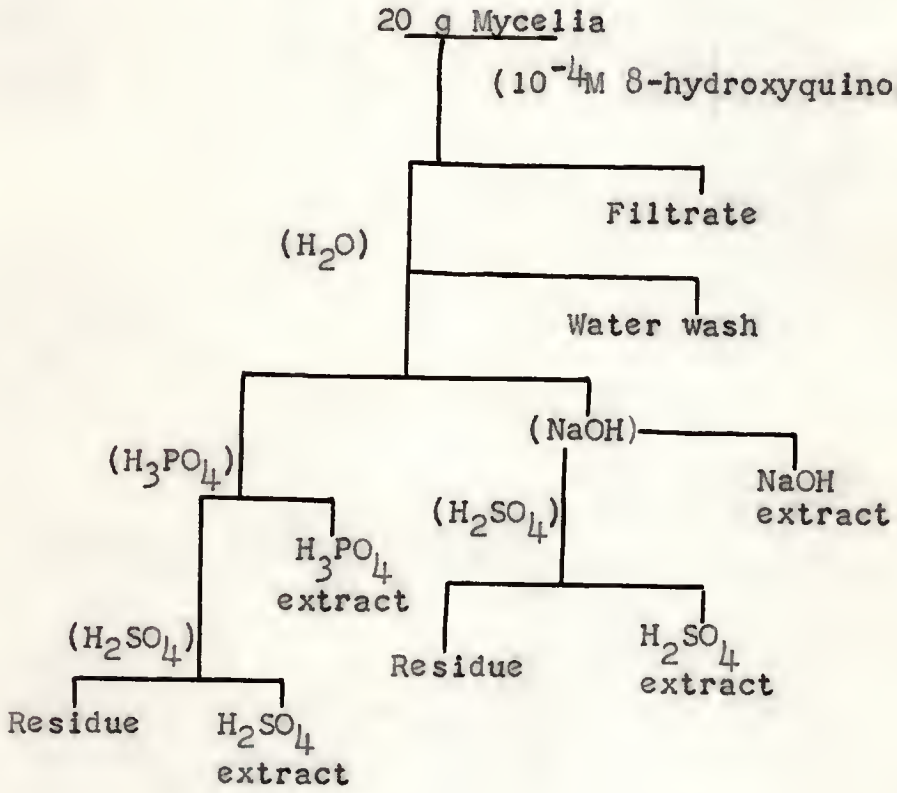
Five 20 g portions of A. niger nycelia, grown in an aereated, shake culture were added respectively to 100 ml of 10^{-4}M 8-hydroxyquinoline-2,4-C^{14} at pH of 3, 4, 5, 6 and 7.5. After 24 hours, in which the cultures were shaken occasionally, the mycelia was filtered and the resulting solutions analyzed for loss of radioactivity due to uptake by the mycelia. Figure 5 shows the loss of 8-hydroxyquinoline (10^{-4}M) vs. pH as determined on the above cultures. This curve is similar to that for 10^{-3}M 8-hydroxyquinoline-9-C^{14} and the slope is about the same.

FIGURE 5


AMOUNT OF 8-HYDROXYQUINOLINE TAKEN UP BY A. niger MYCELIA AT DIFFERENT VALUES OF pH
dps/ml
50
45
40
35
2
3
4
5
6
7
8
pH AT WHICH MYCELIA WAS GROWN

B. Mycelia Fractionation

Once the fact is established that the 8-hydroxyquinoline actually enters the mycelia, as is suggested by these data, it becomes necessary to find the fraction of mycelia into which it is concentrated, as for example, fat, protein, or "carbohydrate" fractions. In order to determine the site of action of the 8-hydroxyquinoline, a general pattern of fractionation was used as follows:



The curves in Figures 6, 7 and 8 show the results of a fractionation of this type on the mycelia treated with 10^{-4}M 8-hydroxyquinoline at five different hydrogen ion concentrations. The mycelia is that used in determining Figure 5. It should be noted from Figure 6 that 25 per cent of the 8-hydroxyquinoline was removed by water washing of the mycelia grown at pH 7, while 21 per cent was washed out from the pH 2 mycelia. This suggests the greater uptake of 8-hydroxyquinoline at pH 7 may be due to rather weak bonds. Figure 7 shows a similar trend - pH 7.5 mycelia giving up the greatest amount of 8-hydroxyquinoline to the cold acid extraction. Both 8-hydroxyquinoline and its chelates are soluble in this acid solution. It is interesting to note that upon boiling in 70 per cent H_2SO_4, Figure 7, no more 8-hydroxyquinoline could be extracted while the residue, consisting of insoluble decomposition products of chitin and proteins, contained a relatively large amount of radioactivity with pH 7.5 mycelia having the lowest specific activity.

The situation as shown in Figure 8 is reversed from the acid extraction insofar as the relative ratio of acid extract vs. pH is concerned. Here the greatest amount of 8-hydroxyquinoline is extracted at pH 5 by NaOH while the residue has the greatest activity at pH 7.5. This

FIGURE 6

AMOUNT OF 8-HYDROXYQUINOLINE WASHED OUT OF MYCELIA

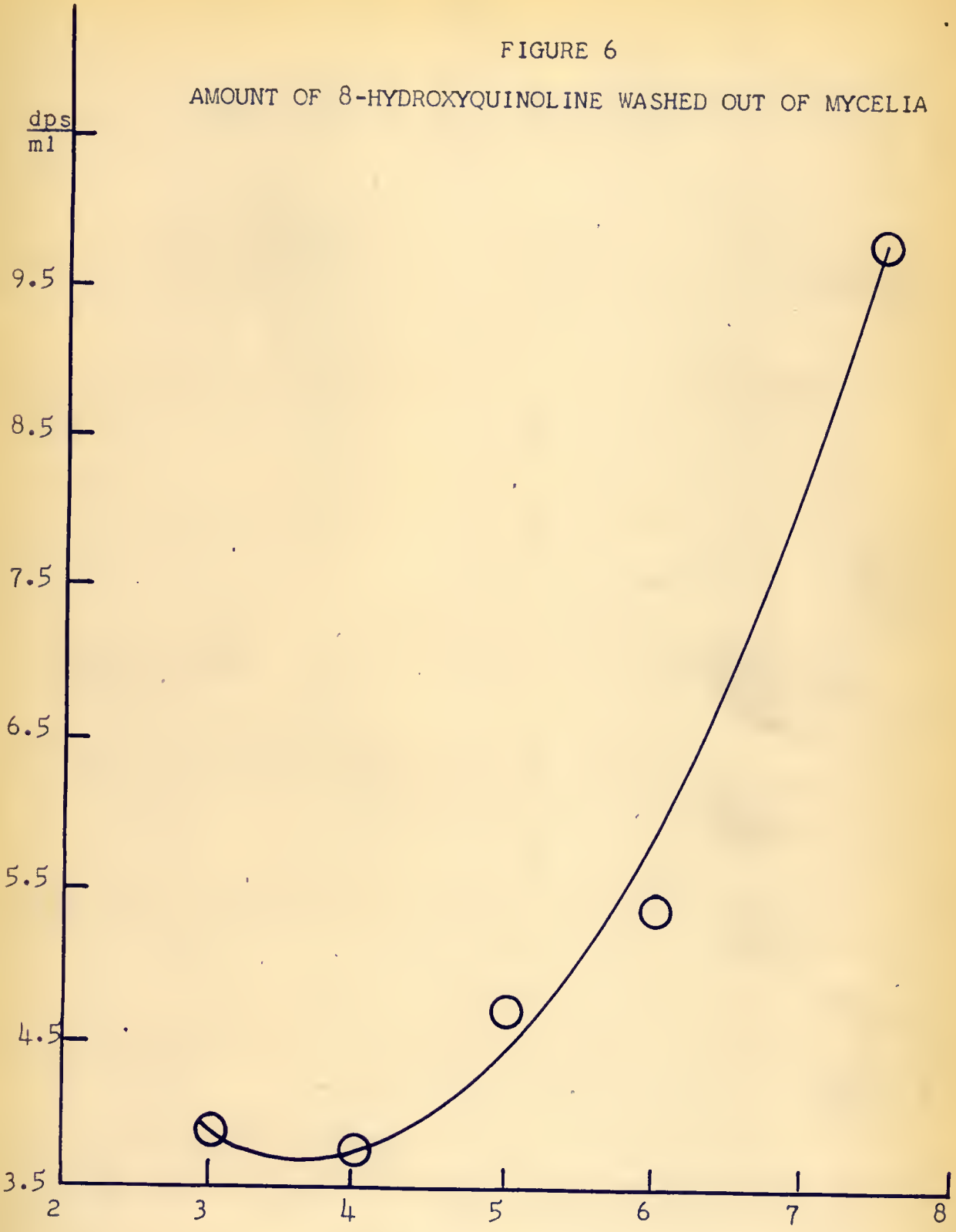


FIGURE 7
PHOSPHORIC ACID EXTRACTS AND RESIDUE

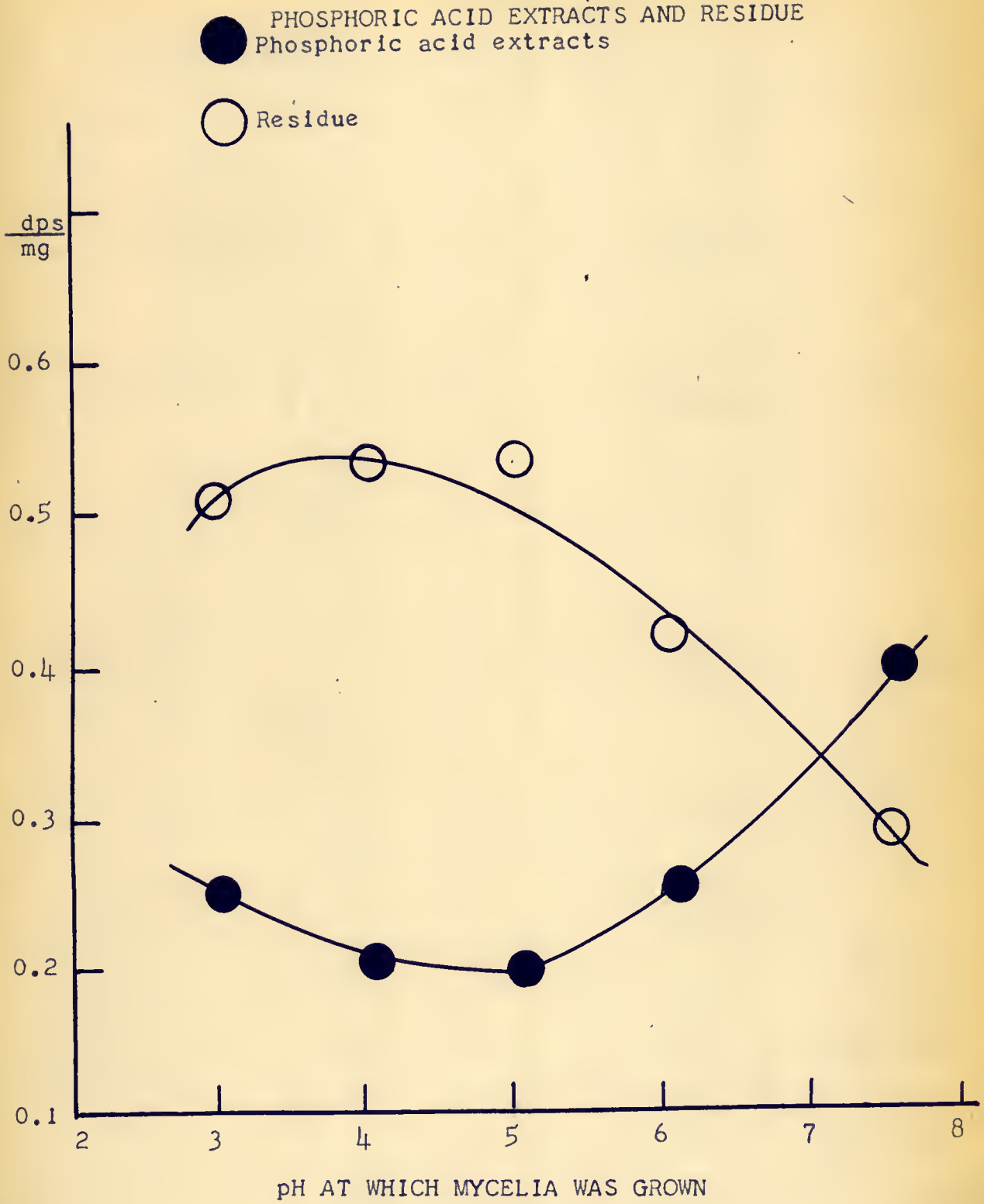
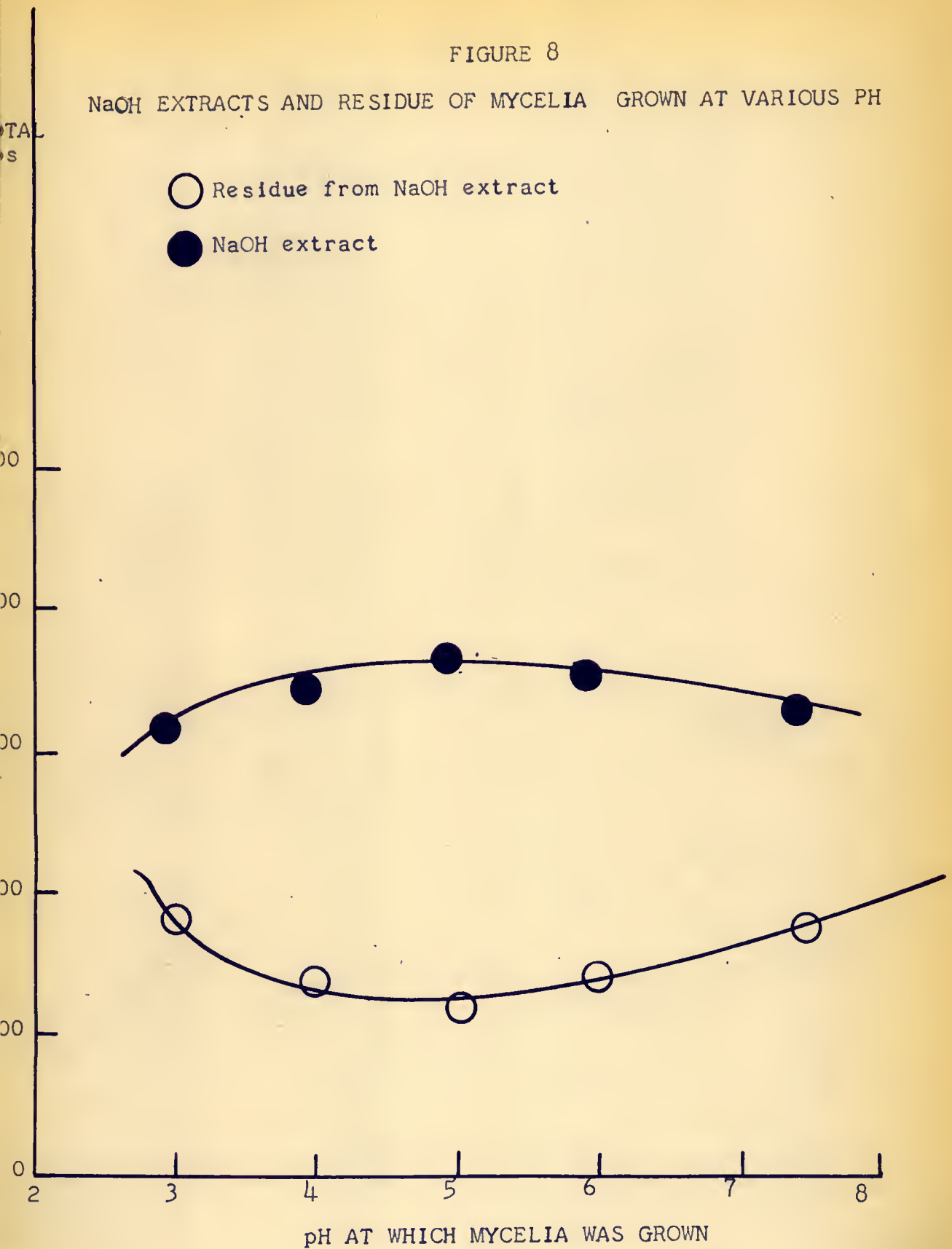


FIGURE 8

NaOH EXTRACTS AND RESIDUE OF MYCELIA GROWN AT VARIOUS PH



indicates that the 8-hydroxyquinoline is more active toward the basic extracts of A. niger mycelia, than toward the acid extracts. It is also noted from Figure 7 and 8 that around pH 5 the slope of the curves change in each case.

An important advantage of the radioactive tracer method is the ability of the experimenter to make a material balance. A typical radioactive balance of this series, choosing pH 7 mycelia, was as follows:

Radioactive Balance

1.	100 ml 10^{-4}M 8-hydroxyquinoline-2,4-C^{14} contains	5,260 dps total activity
2.	8-hydroxyquinoline taken up by mycelia	1,430 dps
3.	8-hydroxyquinoline remaining in solution	3,830 dps
4.	8-hydroxyquinoline washed out	490 dps
5.	H_3PO_4 extract	94 dps
6.	H_2SO_4 extract	0
7.	Residue	144
8.	NaOH extract	330
9.	Residue	170
10.	Total Accounted for	1,228 or 96.1%
11.	Unaccounted for	202
		1,430

C. Radioactive Spores and Mycelia

In an effort to determine if 8-hydroxyquinoline or its metabolic products, if any are formed, enter the growing mycelia and spores, a series of agar plates of mineral salt medium was adjusted to 10^{-4}M with 8-hydroxyquinoline. This allowed very slow growth after a prolonged period (4 weeks) and atypical colonies were formed. Spores were produced which displayed their characteristic color*. The spores were harvested without touching the medium. They were extracted with hot H_3PO_4 and after several hours the black liquid was diluted with formamide and counted. Only qualitative results were desired so the efficiency was considered 1.03 per cent since it was counted in 85 per cent formamide. The spores were found to contain 117 dps equivalent to 0.15 mg of 8-hydroxyquinoline. This evidence indicates the 8-hydroxyquinoline is able to move through the mycelia to the spores since at no time were the spores in direct contact with the medium.

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* A. niger spore color is very sensitive to metals present. If no copper is present, the spores are white, turning black in presence of sufficient copper.

Mineral salt medium (100 ml) at a concentration of 10^{-3}M 8-hydroxyquinoline-9-C^{14} was inoculated with a few spores. After a long (2 weeks) incubation period, a single colony grew in atypical raised fashion. About 95 per cent of the mycelia and spores were above the medium. The growth was removed and the mycelia that had touched the medium was cut off. The mycelia was washed with water, dried and extracted with H_3PO_4. The mixture was diluted with formamide and counted. The mycelia was found to contain 75.5 dps corresponding to .097 mg of 8-hydroxyquinoline-9-C^{14}.

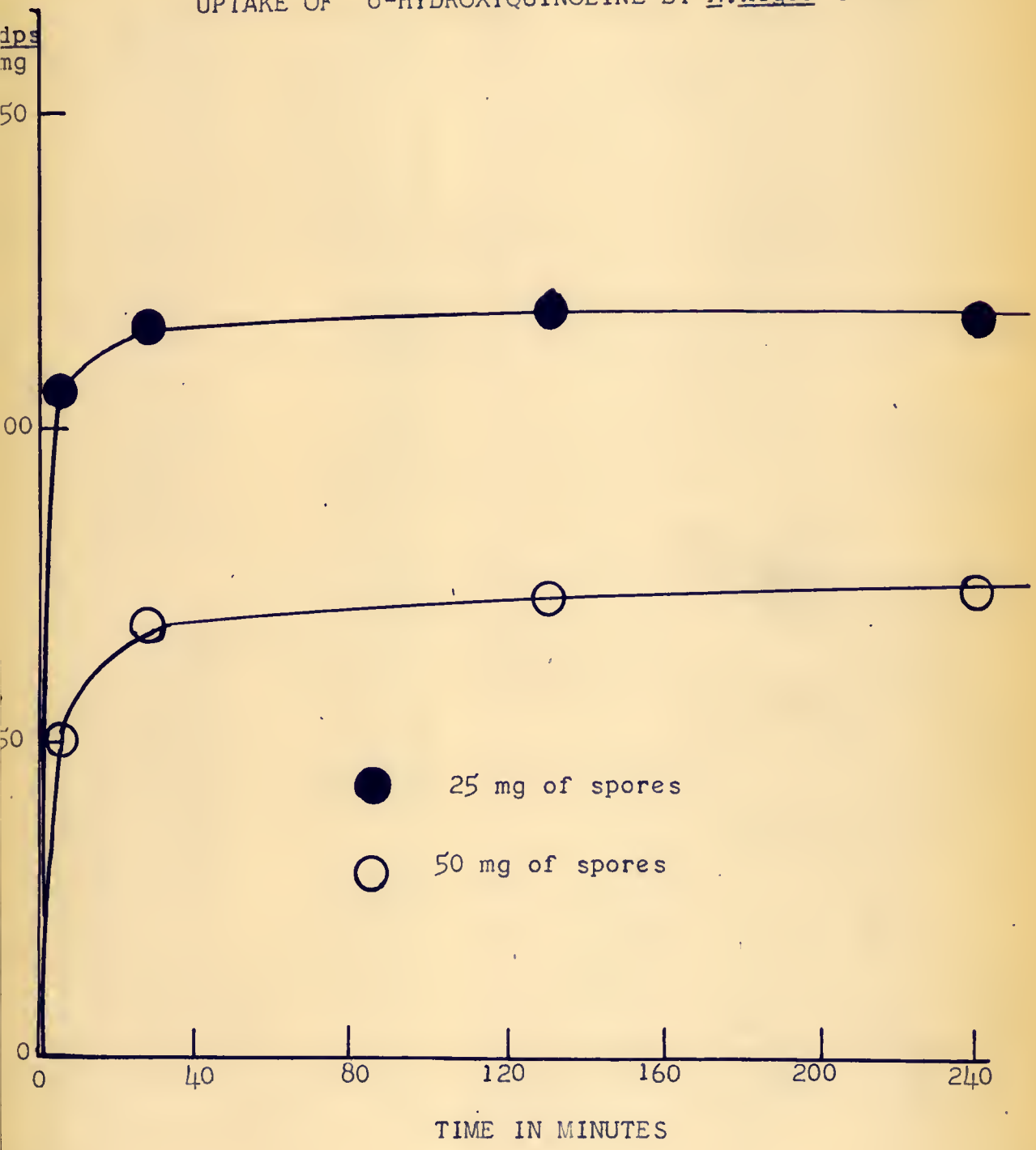
D. Spore Uptake

An important consideration in preservation of material from fungi is the spore. If a fungicide is active against the spore, the most resistant part, it will successfully protect the material. A series of experiments was run in a manner similar to that described by Miller (33) to determine the amount of 8-hydroxyquinoline taken up by the spores of A. niger. Ten ml of 10^{-3}M 8-hydroxyquinoline-9-C^{14} were added to 25 and 50 mg of spores. At time intervals of 5 and 30 minutes and 4 and 30 hours, the mixture was centrifuged

and the 1 ml sample withdrawn for analysis. Figure 9 shows that a large amount of 8-hydroxyquinoline-9-C^{14} is rapidly taken up. This uptake is 10^5 times more per unit weight than with the mycelia. A 10^{-3}M solution of 8-hydroxyquinoline inhibits growth but it would appear from these data that the spores actually require larger doses of 8-hydroxyquinoline to prevent their germination.

FIGURE 9

UPTAKE OF 8-HYDROXYQUINOLINE BY A.niger SPORES



V. STUDIES ON COPPER-8-QUINOLINOLATE

A. Mycelia Uptake

Since copper-8-quinolinolate is more toxic than 8-hydroxyquinoline*, it seems clear that the true toxicity is due to a copper complex of 8-hydroxyquinoline. According to Alberts work on bacteria, he suggests that neither the copper-8-quinolinolate nor the 8-hydroxyquinoline are toxic. According to his hypothesis the copper-8-quinolinolate after entering the cell, breaks down into a charged complex composed of one copper and one 8-hydroxyquinoline molecule. This charged 1:1 complex acts then as the toxic agent. However, if an excess of copper is added to a solution of 8-hydroxyquinoline, favoring the formation of such a complex, the solution shows no great toxicity change. This has been explained on the basis of the difficulty of charged ions in passing through a membrane. With this in mind, experiments were conducted in an effort to determine any difference in uptake of the two complexes. The previous studies with

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* The 8-hydroxyquinoline appears to be only as toxic as the metals present in the substrate and cells.

8-hydroxyquinoline show that a relatively small amount of 8-hydroxyquinoline was taken up on a unit weight basis. It was, however, much larger for the spores than for mycelia. A series of experiments were conducted with A. niger to determine the uptake of various 8-hydroxyquinoline-copper ratios by the mycelia. If one considers the complex equilibria involved between copper and 8-hydroxyquinoline, it is possible to write an equilibrium expression as follows:

$$Cu^{++} + HQ \rightleftharpoons CuQ^{+} + H^{+} \qquad (\log k = 12.0) \qquad (1)$$

$$CuQ^{+} + HQ \rightleftharpoons Q_2Cu + H^{+} \qquad (\log k = 11.0) \qquad (2)$$

or

$$Cu^{++} + 2HQ \rightleftharpoons Q_2Cu + 2H^{+} \qquad (K = 10^{23}) \qquad (3)$$

These formation constants are based on studies in aqueous solutions (34). Irving and Williams (35), Meller and Maley (36) and recently Johnston and Freiser (37) have reported various values from log 12 to 15 for aqueous-dioxane solutions. From this equilibrium it is obvious that copper is present in extremely minute amounts and the formation of copper-8-quinolinolate is greatly favored. It is also clear that this equilibria will be very sensitive to the pH. This has recently been shown to be the case (38). At low pH the equilibrium can be written as follows:

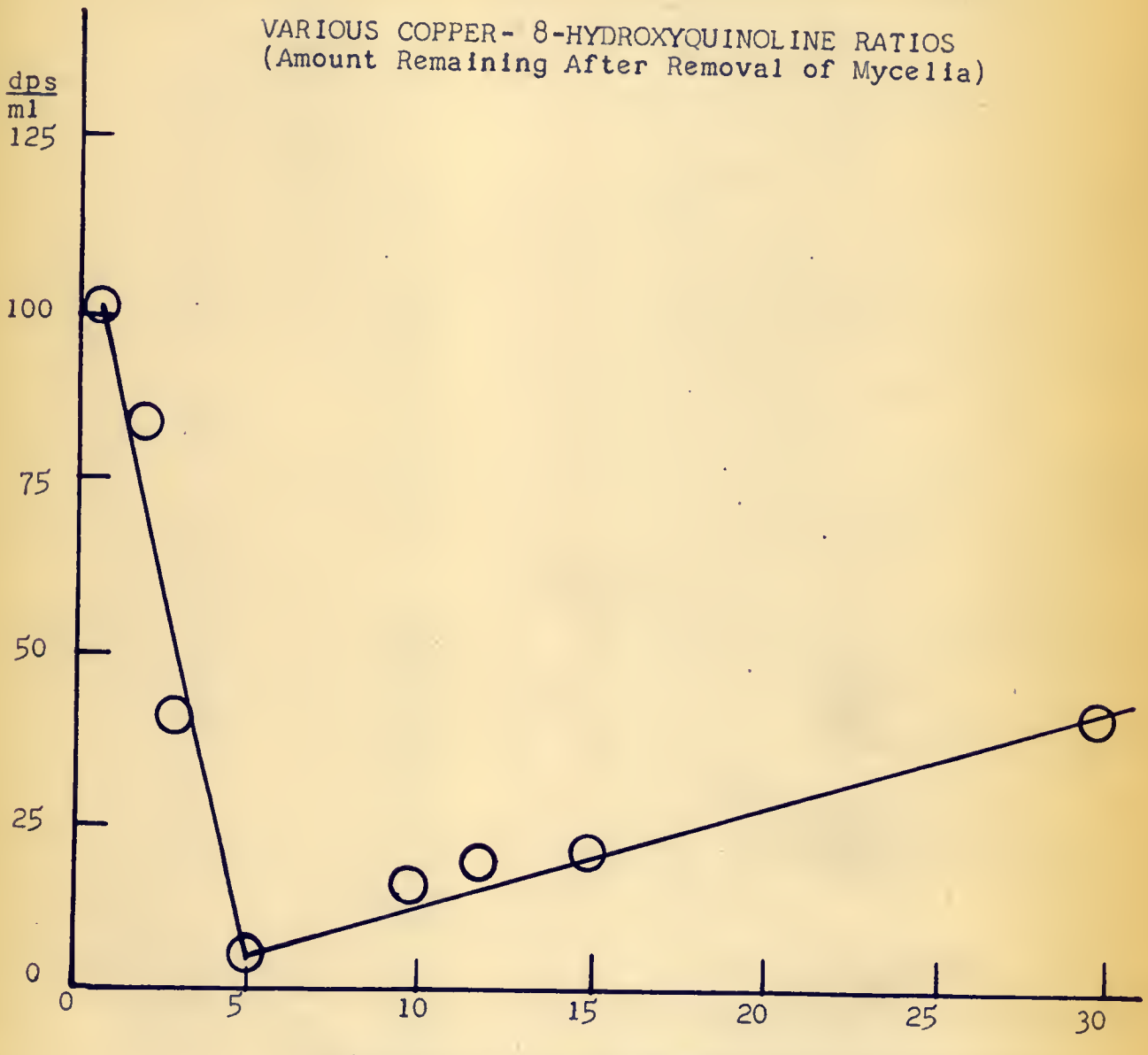
$$Cu^{++} + 2H_2Q^{+} \rightleftharpoons Q_2Cu + 4H^{+}$$

It should be possible to shift the equilibrium in the desired direction by using an excess of either copper or 8-hydroxyquinoline. In the uptake experiments described, a constant amount of 8-hydroxyquinoline was used and copper varied from a deficiency, favoring Q_2Cu, to an excess, favoring CuQ^+.

Equal amounts of mycelia were added to a series of 10 ml samples of 10^{-3}M 8-hydroxyquinoline-9-C^{14}. To each of these flasks different amounts of copper were added. The copper aliquots were 2, 3, 5, 10, 12, 15, and 30 ml of 10^{-3}M copper acetate. The pH of the solution was 5. The resulting range of concentrations would give solutions from 0 to 99 per cent free 8-hydroxyquinoline. The CuQ^+/CuQ_2 ratio varies, depending on the equilibria. At the end of twenty-four hours, the mycelia was filtered and placed in 10 ml of petroleum ether. Figure 10 shows the uptake of radioactive material as observed from this experiment. The maximum shown here corresponds to the highest concentration of the 2:1 complex. It should be noted that the uptake of pure 8-hydroxyquinoline corresponds to the previous experiments for 8-hydroxyquinoline at this particular pH.

FIGURE 10

RADIOACTIVE MATERIAL TAKEN UP BY MYCELIA IN SOLUTIONS OF VARIOUS COPPER- 8-HYDROXYQUINOLINE RATIOS
(Amount Remaining After Removal of Mycelia)



The treated mycelia was extrated with petroleum ether in which only 8-hydroxyquinoline is soluble. Figure 11 shows the amount of 8-hydroxyquinoline removed and this is what one would expect from the uptake curve. As the copper becomes excessive the equilibrium for the formation of 8-hydroxyquinoline is unfavorable. This is borne out by Figure 11. The mycelia was then extracted with a 50 per cent ethanol solution and the extracts analyzed to yield the information shown in Figure 12. The amounts extracted represent about 30 per cent of the total radioactive material.

These results indicate that CuQ_2 is taken up in larger amounts than CuQ^+ or 8-hydroxyquinoline. There is about 90 per cent difference in the uptake of CuQ_2 and pure 8-hydroxyquinoline. They also show, as did previous studies with 8-hydroxyquinoline, that the greatest amount can also be extracted at the maximum point of uptake. It appears that uptake could be a function of lipid solubility, inasmuch as CuQ_2 is more lipid soluble than 8-hydroxyquinoline at the pH used in this experiment. This further implies that the material is entering the cell or at least the lipid layers of the mycelia.

FIGURE 11

RADIOACTIVITY OF PETROLEUM ETHER (30-40°) EXTRACTS OF MYCELIA GROWN AT VARIOUS COPPER-8-HYDROXYQUINOLINE RATIOS

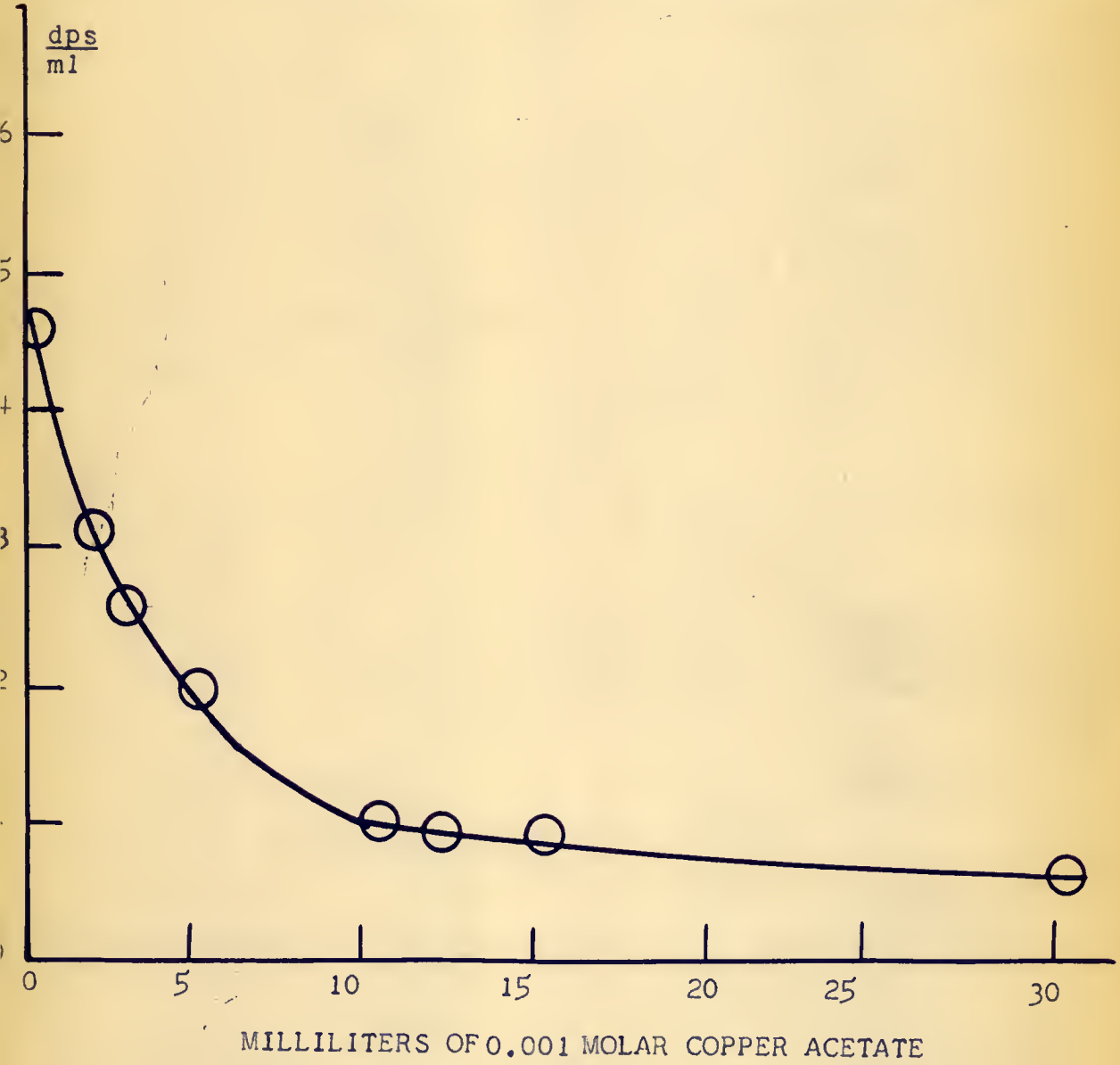
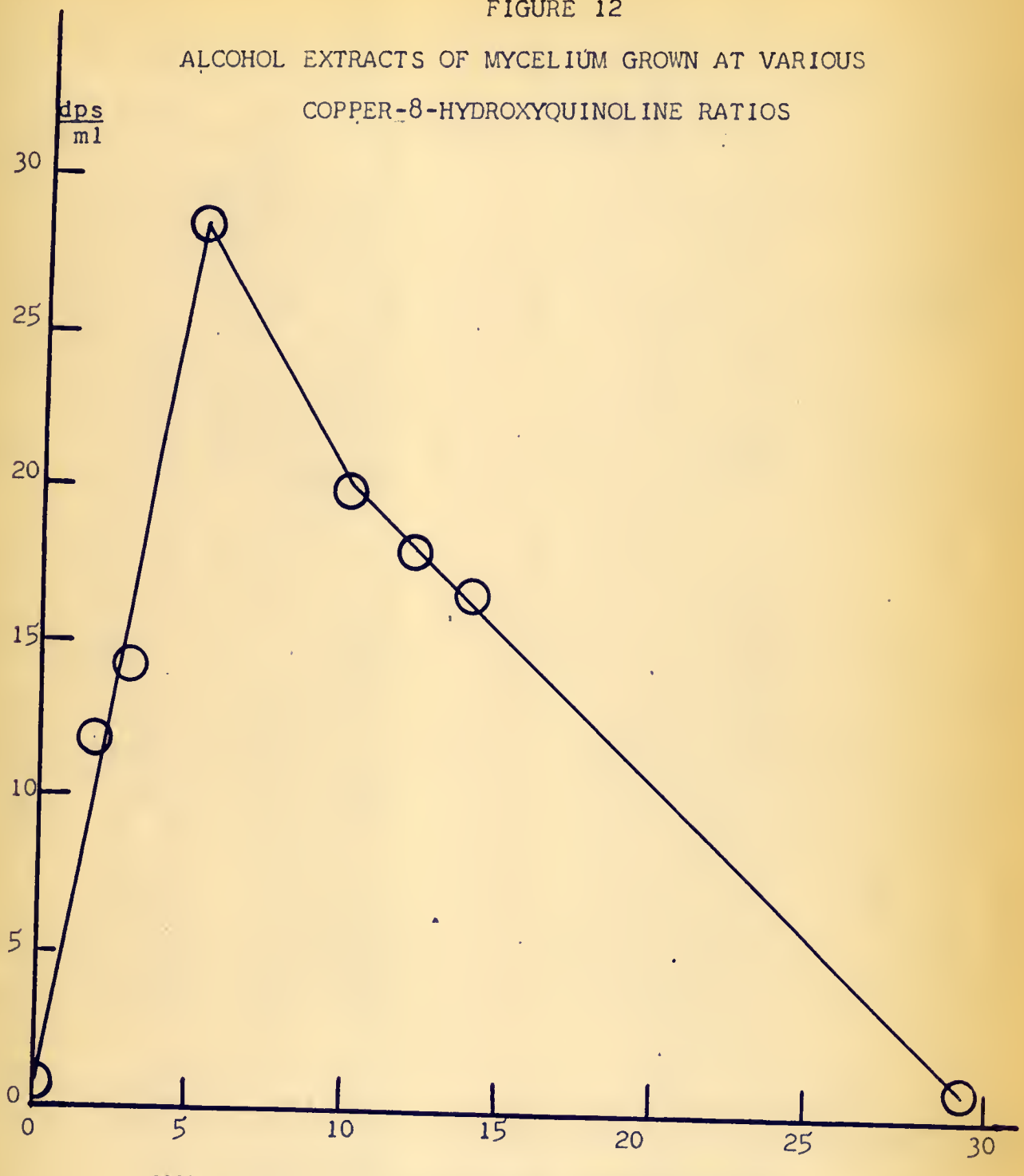


FIGURE 12

ALCOHOL EXTRACTS OF MYCELIUM GROWN AT VARIOUS COPPER-8-HYDROXYQUINOLINE RATIOS



The uptake of the species presumed to be 8-hydroxyquinoline, CuQ^+, and CuQ_2 by A. niger mycelia as has been described was repeated, using a very large excess of copper inasmuch as an increase in copper should insure a more favorable equilibrium for the formation of CuQ^+. It has been suggested by Albert, that due to its lipid solubility, only the CuQ_2 is able to enter the cell. In an attempt to establish this here, a large excess of copper was used so that some CuQ^+ would be present and the uptake should be smaller. In the experiments carried out the uptake results have not agreed with predictions. In four separate experiments the average uptake of 8-hydroxyquinoline, CuQ^+ favored, and CuQ_2 favored solutions was found to be 25 per cent, 85 per cent and 95 per cent respectively. In the light of the small amount of CuQ^+ that would be formed, the difference between the CuQ^+ favored and CuQ_2 favored solutions would be a qualitative measure of the CuQ^+ formed. It is not valid to consider the uptake of CuQ^+ as 85 per cent, actually only the difference (10 per cent) between CuQ_2 and CuQ^+ is significant. This difference may be due to the formation of CuQ^+.

The method used for uptake determination was to place equal amounts of 48 hour A. niger mycelia grown in shake cultures in synthetic medium (Czapek's sucrose, nitrate solution) in a series of flasks containing various cupric chloride-8-hydroxyquinoline concentration ratios buffered at pH 5. The mycelia was noted to take up the color of the chelate at once. The color of the mycelia varied as the concentration of the respective complexes, becoming greener as pure CuQ_2 was approached, and then changing in color with increasing copper concentration. These flasks were then placed on the shaker for 24 hours, after this time the mycelia was filtered and the filtrate evaporated and counted in formamide solution. The difference between the initial count and that for the filtrate was taken as mycelial uptake. The percentage uptake given above are based on initial concentration of 8-hydroxyquinoline, and are found for experiments using 10 g (wet weight) of mycelia in 10^{-3}M 8-hydroxyquinoline solutions.

This high uptake of that copper-8-hydroxyquinoline concentration considered to be $CuQx^+$ is not in agreement with the idea that charged particles are

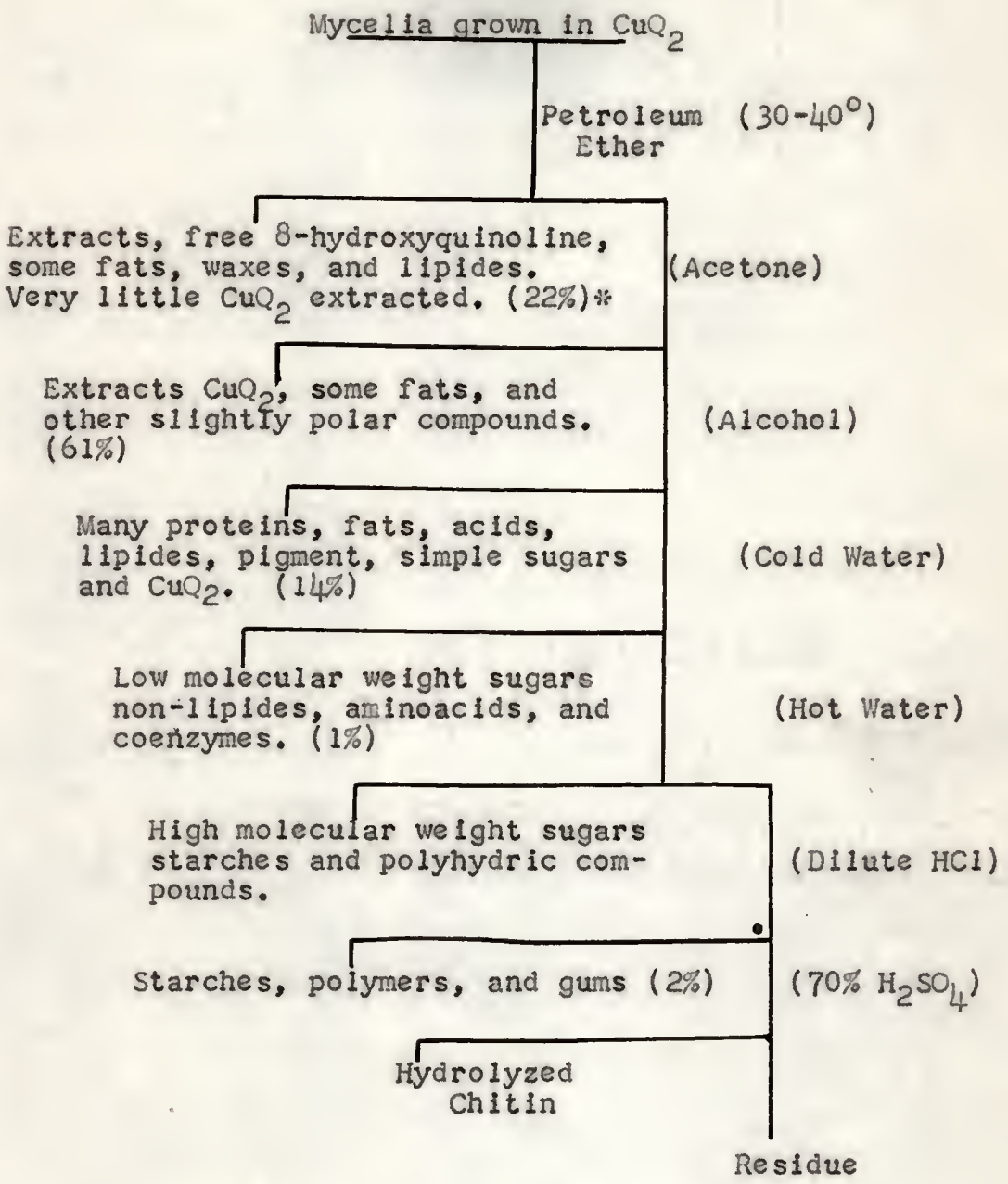
deterred from crossing the lipid membrane considered present in mycelial cells. There are several possible explanations for the results. First, the membrane may possess a negative charge, and surface attraction by electrostatic charges may account for the uptake. Second, the membrane may simply extract the CuQ_2 present, and shift the equilibrium toward the formation of more CuQ_2. The third and most probable reason for this high uptake is due to the fact that only a very small amount of CuQ^+ is formed, the major constituent being CuQ_2, as suggested in the previous paragraph.

The uptake of CuQ_2 has been determined for A. niger on a wet and dry weight basis. Washed mycelia of A. niger (50 grams wet or 1.4 grams dry) was shaken in a 10^{-3}M $CuCl_2$ solution after the addition of 8-hydroxyquinoline (5×10^{-4}M CuQ_2 mixture of 136 dps/ml for a total activity of 0.73μc). After the mycelia had been filtered, the used medium had an activity of 39.2 dps/ml or a total activity of 0.19μc. This corresponds to a 75 per cent uptake or 0.38 μc/gram dry weight. This represents 1.55 mg CuQ_2/gram dry weight or a 0.15 per cent pickup.

B. Mycelia Fractionation

The mycelia grown in the uptake experiments was fractionated to determine the distribution of the copper-8-quinolinolate in the material. The fractionation of mycelia was carried out by growing a relatively large amount of mycelia and treating it with 5×10^{-4}M solutions of copper-8-quinolinolate-9-C^{14} at pH 4.5. The mycelia was fractionated and attempts were made to identify the radioactive materials. The mycelia extracts from uptake experiments were chromatographed to identify the species present. Whatman No. 1 filter paper (one inch strips) were used with a series of developing solvents ranging from butanol-saturated water to chloroform-ethylene chloride mixtures. Butanol-saturated water was found to give the best R_f value (ca. 0.52) for 8-hydroxyquinoline. Free 8-hydroxyquinoline was found in the petroleum ether extracts. It has recently been shown by Hutchison (38) that under certain conditions, appreciable amounts of the total copper-8-quinolinolate in a system of water-chloroform, can be found as free 8-hydroxyquinoline.

After filtration, the mycelia was extracted (Soxhlet) for 48-hours with 30-40° petroleum ether. 8-hydroxyquinoline is readily soluble in petroleum either while copper-8-quinolinolate is quite insoluble (continuous extraction of 17.5 mg of the pure chelate for 72 hours removed less than 3 mg). The petroleum ether extract was a mixture of fatty and crystalline material. Pure crystalline 8-hydroxyquinoline was isolated from this fraction. Further fractionation with alcohol, water, and acid was carried out, and each fraction was counted in formamide solution, or as solid material. Since activity was found in all the fractions of this mycelia, only a general picture could be obtained from this approach. Fractionation by extraction with different solvents allows only partial fractionation in a very complex system such as the mycelia of fungi. The fractionation and determination of the site of the radioactive 8-hydroxyquinoline and its copper complexes was carried out by the following scheme of separation:


Mycelia grown in CuQ_2
Petroleum Ether (30-40°)
Extracts, free 8-hydroxyquinoline, some fats, waxes, and lipides. Very little CuQ_2 extracted. (22%)*
(Acetone)
Extracts CuQ_2, some fats, and other slightly polar compounds. (61%)
(Alcohol)
Many proteins, fats, acids, lipides, pigment, simple sugars and CuQ_2. (14%)
(Cold Water)
Low molecular weight sugars non-lipides, aminoacids, and coenzymes. (1%)
(Hot Water)
High molecular weight sugars starches and polyhydric compounds.
(Dilute HCl)
Starches, polymers, and gums (2%)
(70% H_2SO_4)
Hydrolyzed Chitin
Residue

*The percentages are based on the total radioactivity of the mycelia.

Further work was carried out on the fractionation of mycelia previously treated with copper-8-quinolinolate. In an effort to determine if this chelate was undergoing a breakdown, mycelia of A. niger was treated with sublethal concentrations and the resulting mycelia fractionated. The various fractions were assayed for carbon-14. The experiment yielded interesting information on the distribution of carbon-14 within the mycelia. Actively growing mycelia (net weight 125 g) was treated with 8-hydroxyquinoline-2,4-C^{14} (8 mg) with a specific activity of 0.1375 μc/mg. The total activity was 1.11 μc. To the mycelia, 5.5 ml of 10^{-2}M $CuSO_4$ was added to form the copper-8-quinolinolate.

The mycelia was allowed to remain in this solution for 24 hours with constant shaking. A small portion of mycelia was removed and plated on agar. It grew slowly at first, and finally at a normal rate.

The mycelia was filtered and the filtrate counted to determine the uptake. The results are as follows:

Initial activity 1.10μc

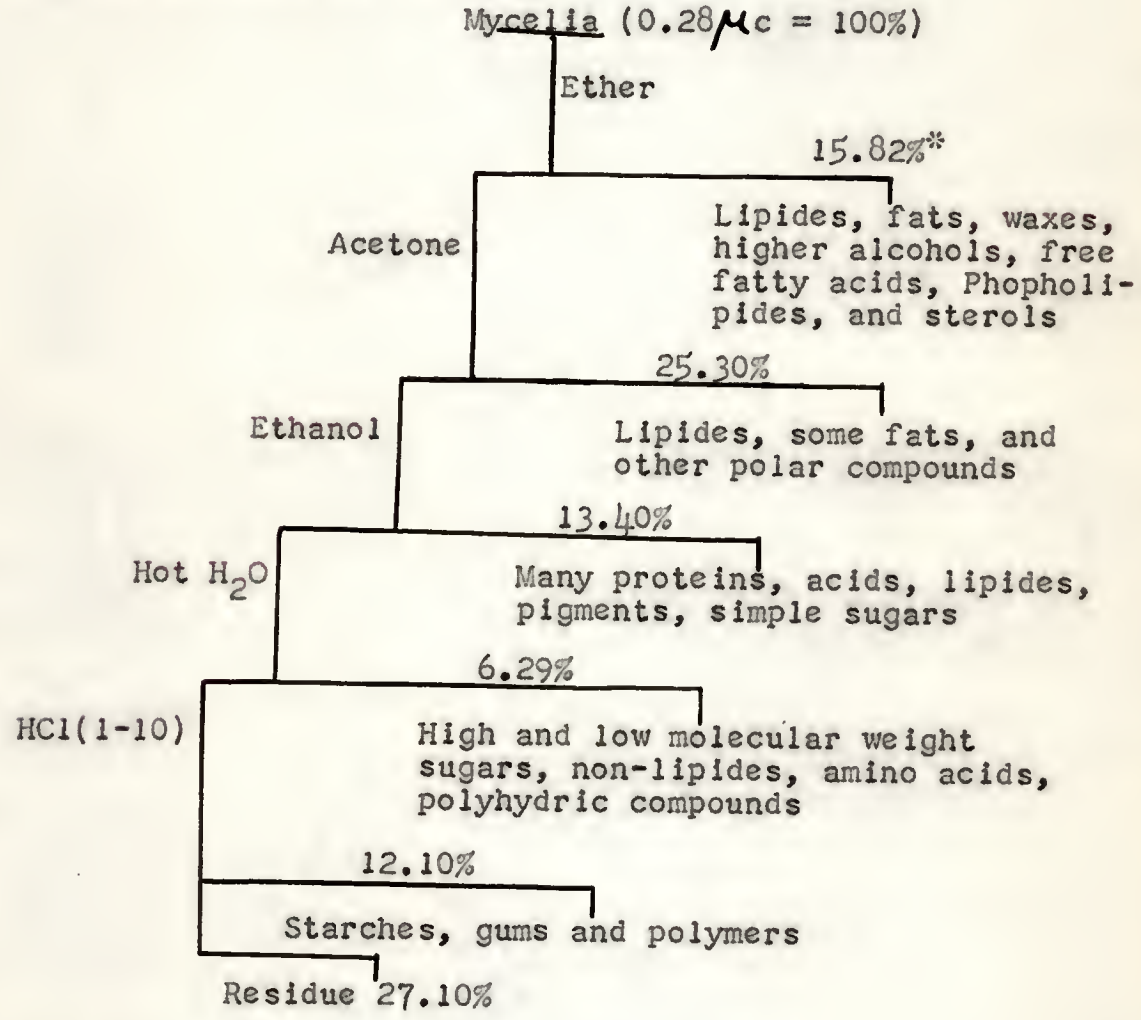
Filtrate activity 0.82μc

Activity in mycelia 0.28μc = 10,400 dps

Weight of dried mycelia 11.5061 g

Uptake = 2.43×10^{-2} μc/g

The mycelia was fractionated by extraction with various solvents as follows:



*Per cent of total activity in mycelia.

TABLE IV

AMOUNTS OF EXTRACTED MATERIAL AND THEIR CARBON-14 ACTIVITIES

Extraction liquid	Milligrams extracted	Per cent of total weight	Specific activity dps/mg	Total activity dps	Per cent of total activity
Ether	212	1.8	7.51	1,590	15.82
Acetone	2,113	18.3	1.21	2,542	25.30
Ethanol	1,101	9.5	1.23	1,350	13.40
Water (hot)	988	8.5	0.64	632	6.29
HCl (1-10)	1,069	9.3	1.14	1,219	12.10
Residue	6,001	56.0	0.42	2,718	27.10
Amount recovered	11,484			10,051	
Initial amount	11.5061			10,320	

99 per cent recovery of weight

97.5 per cent recovery of activity

C. Spore Uptake

A parallel series of experiments with the mycelia uptake were conducted using spores in place of mycelia. The spores were added to solutions of various copper-8-hydroxyquinoline concentrations and after a 24 hour period, they were filtered and the solid spores and the filtrate were analyzed for carbon-14. The results were similar to the previous ones using mycelia, except that more free 8-hydroxyquinoline was taken up by the spore than by the mycelia. This agrees with previous studies on free 8-hydroxyquinoline.

A second series of experiments on uptake studies of spores were carried out to get a comparison with the mycelia. The spores were suspended in the solution of toxic materials for 24 hours. At this time they were centrifuged and the liquid, free from spores, was evaporated and counted. The following results were obtained:

TABLE V

A. NIGER SPORE UPTAKE

Ratio of 8-hydroxy-quinoline to copper	Uptake (μc/gm)	Concentration
1:0	55×10^{-2} μc/gm	5 ml 8-hydroxyquinoline-2,4-C^{14} (10^{-3}M)
2:1	138×10^{-2} μc/gm	" + 2.5 ml 10^{-3}M $CuSO_4$
1:10	97×10^{-2} μc/gm	" + 5 ml 10^{-1}M $CuSO_4$

The spores were washed with ethanol, acetone, ether, and water, but they retained activity as shown below.

TABLE VI

RESIDUAL ACTIVITY IN SPORES

Ratio of 8-hydroxy-quinoline to copper	Concentration	Specific activity
1:0	5 ml 10^{-3}M 8-hydroxy-quinoline	7.1×10^{-2} μc/gm
2:1	" + 2.5 ml 10^{-3}M $CuSO_4$	11.9×10^{-2} μc/gm
1:10	" + 5 ml 10^{-1}M $CuSO_4$	34.1×10^{-2} μc/gm

As it has previously been noted the spores take up relatively large amounts of copper-8-quinolinolate and 8-hydroxyquinoline compared to mycelia. A new method for study of spore uptake and removal of toxicant has been worked out.

A series of experiments have been run to determine the uptake and ease of removal of 8-hydroxyquinoline with various concentrations of copper. A. niger spores were placed in 50-ml tubes filled with the solutions shown in Table VII.

TABLE VII

A. NIGER SPORE UPTAKE

Number	Spores (mg)	8-hydroxyquinoline-9-C^{14} (mM)	$CuCl_2$ (mM)
1	41.2	.01	0.
2	32.8	.01	.005
3	37.1	.01	.01
4	42.3	.01	.1

The spores were allowed to stand 24 hours at 30°C. The spores were suspended in the solution and filtered onto paper disks of exactly 2.895 cm^2. The disks were dried and counted. The spores were then treated, on the paper disks, with ethanol (10 ml of

95 per cent) after which they were dried and recounted. This was done again using H_2O(20 ml). Table VIII gives the tabulated results from this experiment.

TABLE VIII

RESIDUAL ACTIVITY IN SPORES

No.	Spores (mg)	Specific Activity	Activity after Ethanol wash	Activity after water wash
1	5.8	14.0 dps/mg	4.4 dps/mg	3.9 dps/mg
2	4.9	32.4 dps/mg	12.2 dps/mg	9.4 dps/mg
3	5.6	10.5 dps/mg	10.4 dps/mg	8.4 dps/mg
4	6.4	14.7 dps/mg	14.5 dps/mg	12.8 dps/mg

It is interesting to note the large amount of carbon-14 activity remaining in 3 and 4 after both treatments. This indicates either the entrance of the material into the spores, or a very tight bonding to the spores. The per cent of carbon-14 activity remaining on the spores, after treatment with water and ethanol, was 28 per cent, 81 per cent, and 87 per cent, respectively, for numbers 1, 2, 3 and 4, from the above Table VIII. This method of study will allow extensive studies to be carried out with various types of spores and chemical agents.

D. Apparent Breakdown of the Chelate

In an effort to check the possibility of a decomposition to CO_2 of either 8-hydroxyquinoline or its copper complexes, an experiment was carried out so all gaseous products from the growth of A. niger mycelia would be collected.

The apparatus was a flask fitted with a ground glass joint, allowing for the entrance of CO_2 into a KOH scrubber bottle.

Actively growing freshly washed A. niger mycelia was first placed in three flasks, as described above. 8-Hydroxyquinoline-9-C^{14} (10 ml of 10^{-3}M) with a total activity of 0.028 μc was added to all three. Copper chloride (10 ml of 5×10^{-3} M and 10 ml of 10^{-3}M) was added to the other two in amounts to give exactly enough and an excess copper for the formation of CuQ_2. Carbon dioxide free air was prepared by passing it through 2N KOH. The CO_2 produced by the mycelia was collected in 0.1N NaOH. Table IX shows the amount of CO_2 produced, expressed as Na_2CO_3. The Na_2CO_3 was determined by the method of Willard and Furman (40).

TABLE IX

RADIOACTIVE CARBONATE PRODUCED

Number	Ml 10^{-3}M 8-hydroxy-quinoline	Ml 10^{-3}M $CuCl_2$	Mg of Na_2CO_3 produced
1	10 ml	0	3650 mg
2	10 ml	5 ml	4500 mg
3	10 ml	10 ml	4250 mg

For determination of carbon-14 activity, one-ml samples were precipitated with a $Ba(OH)_2$-$BaCl_2$ mixture. These, however, gave no indication of any activity. For cases of very low activity, the vibrating reed electrometer is essential. Samples (1 ml) of the NaOH-Na_2CO_3 mixture were treated with acid to liberate the CO_2 which was collected in ionization chambers and the drift rate was determined. The background drift rate is 0.29 millivolts/second, and 0.1543 millivolts/second over the background drift is equal to 1 dps. Table X shows the results of the three samples of CO_2.

The amount of activity is small, but it is almost twice the drift background. Under the experimental conditions used, the activity can be contributed only to $C^{14}O_2$. Since the activity is so low, further work was done in which 8-hydroxyquinoline-2,4-C^{14} was used.

TABLE X

VIBRATING REED DATA

(1 dps = 0.1543 mv/sec)

Number	Drift Rate	Background Drift	Total Activity of CO_2
1	0.444 mv/sec	0.290 mv/sec	100 dps
2	0.485 mv/sec	0.290 mv/sec	127 dps
3	0.527 mv/sec	0.290 mv/sec	154 dps

A second experiment to determine the decomposition of 8-hydroxyquinoline and its copper complexes was carried out, as described above. Vigorously growing (48 hours) A. niger mycelia was placed in 250 ml flasks fitted with a ground glass joint, allowing for the entrance of CO_2 free air and exit of the CO_2 containing air into a NaOH (100 ml of 0.8N) scrubber solution. Table XI shows the concentrations of 8-hydroxyquinoline and copper used and the amounts of CO_2 produced by the culture. For determination of the carbon-14 present, one-ml samples of scrubber solution were treated with acid to liberate the CO_2. The dried CO_2 was collected in ionization chambers, and the rate of drift in mv/sec was determined. The background rate (after cleaning insulation) was 0.139 mv/sec. One dps is equal to a rate of 0.1543 mv/sec over the background.

TABLE XI

VIBRATING REED DATA

Number	1	2	3
mM 8-hydroxy-quinoline-2,4-c^{14}	.01	.005	.01
mM $CuSO_4$	0.	.0025	.1
Total activity	5,291 dps	2,645 dps	5,921 dps
Ml of scrubber	100 ml	100 ml	100 ml
Milligrams of Na_2CO_3 produced	1,741 mg	2,210 mg	2,110 mg

(Specific activity of 8-hydroxyquinoline-2,4-c^{14} - 5,291 dps/mg)

Table XII shows the results from the three samples as determined by the vibrating reed.

TABLE XII

VIBRATING REED DATA

(1 dps = 0.1543 mv/sec)

No.	Drift Rate	Background Drift	Total Activity
1	1.354 mv/sec	0.139	786 dps
2	0.849 mv/sec	0.139	460 dps
3	0.889 mv/sec	0.139	486 dps

The amounts of activity were greater than those found in previous work (4 to 7 times). The high activity noted for No. 1 might be explained on the basis of the growth after the addition of the toxic compounds. The vigorous growth was continued in No. 1 while Nos. 2 and 3 were inhibited and finally killed by the mixture used. Heavy spore formation was observed in the pure 8-hydroxyquinoline culture (No. 1). The over-all higher activity was expected due to the higher 8-hydroxyquinoline-2,4-C^{14} (5,291 dps/mg) activity as compared to 8-hydroxyquinoline-2,4-C^{14} (1014 dps/mg). These results are more significant than the ones previously described, for the drift rate was 8 to 10 times the background. It seems clear from these experiments that 8-hydroxyquinoline is undergoing some type of decomposition to CO_2.

VI. SOLUBILITY BEHAVIOR OF 8-HYDROXYQUINOLINE AND COPPER-8-QUINOLINOLATE

A. Water-Olive Oil Distribution

To further the uptake and equilibrium studies the distribution of CuQ_2 and 8-hydroxyquinoline between water and olive oil was studied. The distribution of a dissolved substance between two immiscible liquids may be treated according to the laws of mass action for reversible physicochemical reactions as follows:

(C_1) CuQ_2 (dissolved in water) $\rightleftharpoons$ CuQ_2 (dissolved in olive oil) (C_2)

Where $K = \frac{CuQ_2 \text{ Olive Oil } (C_2)}{CuQ_2 \text{ Water } (C_1)}$, is true if the active masses are proportional to concentrations. In an initial experiment using 8-hydroxyquinoline-9-C^{14} in mixture with copper-8-quinolinolate-9-C^{14} at different ratios, the following results were obtained for the water-olive oil system:

Number 1 (At Equilibrium)	Number 2 (At Equilibrium)
0.013 mMoles CuQ_2	0.003 mMoles CuQ_2
0.004 mMoles 8-hydroxyquinoline	0.024 mMoles 8-hydroxyquinoline
CuQ_2; 8-hydroxyquinoline ratio	CuQ_2; 8-hydroxyquinoline ratio
4 : 1	1 : 8

Using activity (Carbon-14) as effective concentration,

K = 395.2 dps/17.3 dps K = 399 dps/12.8 dps

K = 23 K = 31

These results agree with the uptake experiments. They show that free 8-hydroxyquinoline must have a fairly favorable K inasmuch as the Number 2 value is eight units greater than Number 1 and the ratio of CuQ_2 is lower. It should be pointed out that the use of $C_2/C_1 = K$ may be invalid in some cases, for the actual stoichiometric distribution ratio will depend on the degrees of dissociation or association of the CuQ_2 in the two liquid phases (39). In these calculations it is assumed that the CuQ_2 will remain essentially as a single neutral molecule in both water and olive oil.

In order to determine the effect of copper on this equilibrium by other experimental evidence, solutions of various copper-8-hydroxyquinoline ratios were mixed, allowed to precipitate and the precipitate was dried and weighed. From these experiments the following data were obtained. The solutions were buffered at pH 4.

Ratio of copper to 8-hydroxyquinoline	Milligrams of Precipitate
Series 1	
1:1	20.2 mg
1:2	25.2 mg
Series 2	
2:1	7.1 mg
1:1	7.4 mg
1:2	8.3 mg

This information indicates that increasing cupric ion concentration lowers the amount of precipitate formed. Whether this lowering is due to formation of CuQ^+ or to difference in solubilities of chelates at the various salt concentrations must be determined by other methods.

B. Water-Carbon Tetrachloride Distribution

An experiment has been carried out to show the formation of a copper-8-hydroxyquinoline complex that is soluble in water. A water carbon tetrachloride system was used due to its similarity to the lipid-aqueous system of living cells. Carbon tetrachloride (10 ml), 8-hydroxyquinoline-9-C^{14} (10 ml of 10^{-3}M), and $CuCl_2$ (various concentrations) were mixed in separatory funnels and allowed to stand for 24 hours at 20°C. Table XIII shows the concentration of the various mixtures. The total carbon-14 activity of the 10 ml of 8-hydroxyquinoline was 0.028 μc. The aqueous solution was buffered at pH 4.

TABLE XIII

CARBON TETRACHLORIDE DISTRIBUTION DATA

Number	Millimoles 8-hydroxyquinoline	Millimoles $CuCl_2$	Ml of CCl_4
1	0.01	0	10
2	0.01	0.005	10
3	0.01	0.01	10
4	0.01	0.1	10
5	0.01	1.0	10
6	0.01	10.0	10

After standing, 2 ml samples of both the CCl_4 and water layer were removed, placed in stainless steel

dishes, evaporated and counted. Table XIV shows the observed activities of carbon-14 in the two phases.

TABLE XIV

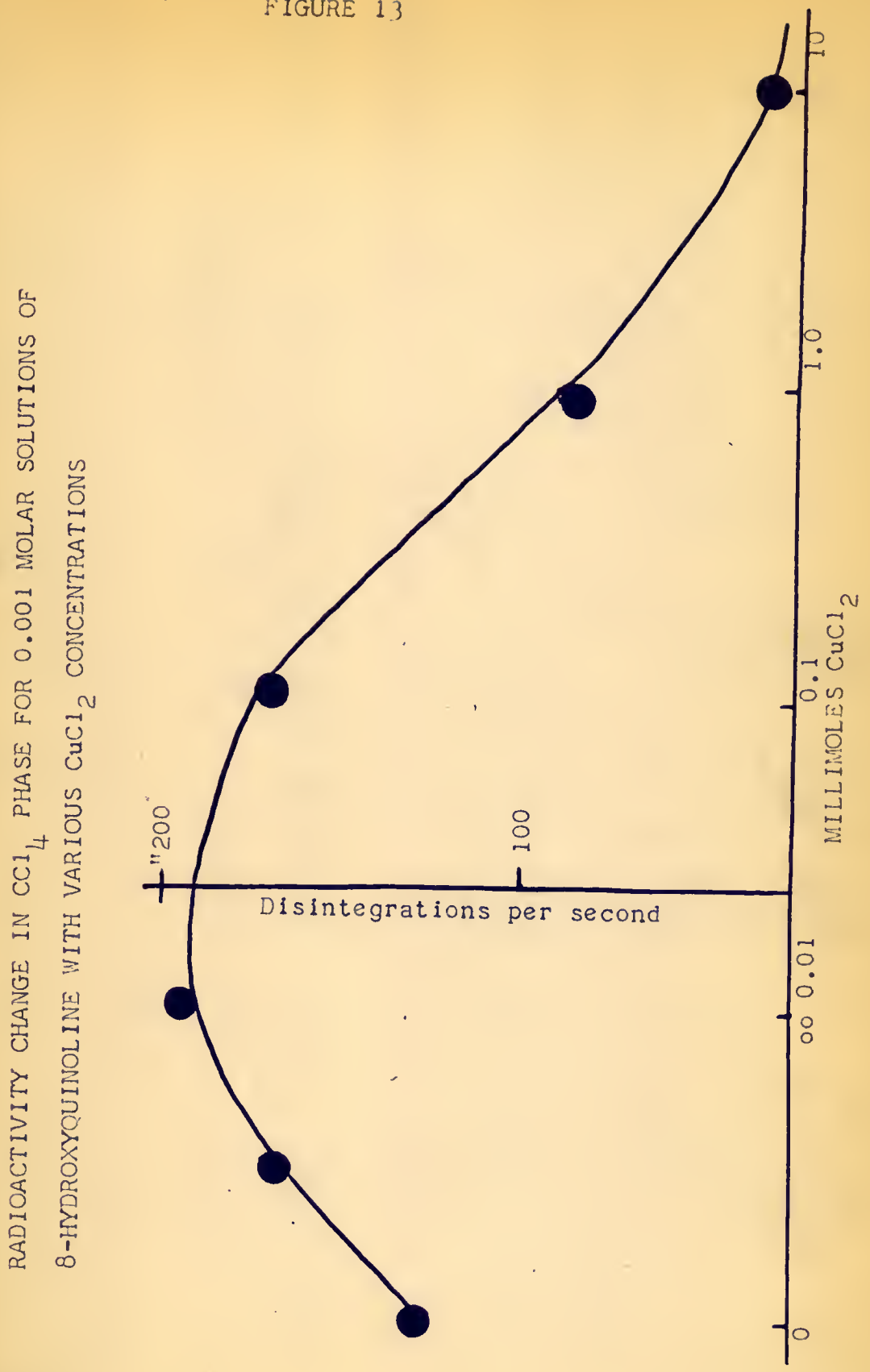
DISTRIBUTION COUNTING DATA

No.	CCl_4 layer dps/ml	Water layer dps/ml	Total activity	Per cent accounted for
1	13.4	42.5	989	97.5
2	17.3	11.3	399 (396) solid	*39.3 (98.0)
3	19.7	18.5	567 (442) solid	*56.0 (99.5)
4	17.7	41.4	1006	99.3
5	9.2	46.0	1012	99.8
6	4.9	47.6	1003	99.1

*In Nos. 2 and 3 the solubility of the CuQ_2 was exceeded and it precipitated to form a solid phase between the CCl_4-H_2O interface. This was collected and the activity determined, as the bracketed values show. The results are included, inasmuch as the difference in solubility in the two phases was the major interest.

Figures 13 and 14 show the change in solubility with increased copper concentration. From these graphs it is clear that great excesses of copper shift the $CuQ_2 + Cu \rightleftharpoons 2CuQ^+$ equilibrium to the right. It is clear also from these data that considerable amounts of 8-hydroxyquinoline-copper complex are present in both aqueous

FIGURE 13


RADIOACTIVITY CHANGE IN CCl_4 PHASE FOR 0.001 MOLAR SOLUTIONS OF 8-HYDROXYQUINOLINE WITH VARIOUS $CuCl_2$ CONCENTRATIONS
"200
100
Disintegrations per second
0
0.01
0.1
1.0
10
MILLIMOLES $CuCl_2$

RADIOACTIVITY CHANGE IN THE WATER PHASE FOR A 0.001 MOLAR SOLUTION OF 8-HYDROXYQUINOLINE WITH VARIOUS $CuCl_2$ CONCENTRATIONS

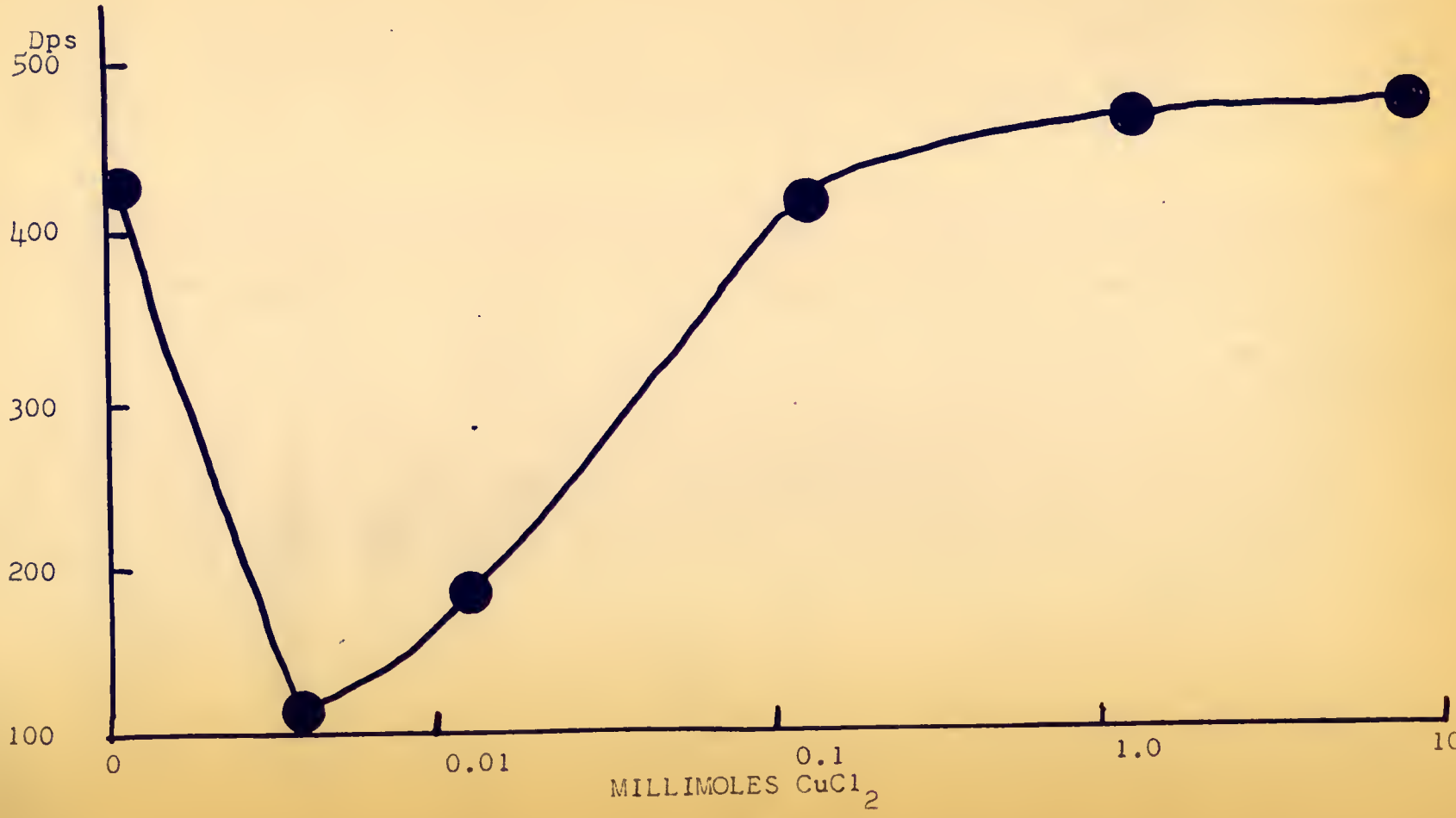


FIGURE 14

and non-aqueous systems. At the peak for the non-aqueous system, 20 per cent of the complex is in the CCl_4 layer, 10 per cent in the water layer, and 69 per cent as solid. The water system, however, at its peak, possesses 94 per cent of the complex, while the CCl_4 system holds only 4.8 per cent. This shows that the CuQ^+ complex can occur in higher concentrations in an aqueous phase than CuQ_2 can in a lipid phase. This may be an important force in the living cell equilibria.

C. Water-Octadecanol Distribution

In an effort to conduct an experiment that represents, as nearly as possible, the probable situation at a cell membrane, octadecanol and water were used to furnish the proper type of interface. Octadecanol (10 ml), 8-hydroxyquinoline-2,4-C^{14} (1.45 mg), and $CuSO_4$ (various amounts) were mixed in separatory funnels and allowed to stand for seven days at 30°C. Table XVI shows the concentration of the various mixtures used. The specific activity of the 8-hydroxyquinoline was 5,291 dps/mg.

TABLE XVI

OCTADECANOL DISTRIBUTION DATA

Number	mM 8-hydroxy-quinoline	mM $CuSO_4$	Ml H_2O	Ml Octadecanol
1	10^{-2}	0.	10	10
2	10^{-2}	.005	10	10
3	10^{-2}	.01	10	10
4	10^{-2}	.5	10	10
5	10^{-2}	5.0	10	10

The mixtures shown in Table XVI were mixed and shaken at various time intervals for seven days. After two days of standing, the water layer was drawn off and two samples from each mixture were placed in aluminum

dishes of 5.0 cm diameter, evaporated and counted. The efficiency of the counting was determined by preparing calibration curves of constant radioactivity of 8-hydroxyquinoline against various amounts of copper. In this experiment, the solutions were not buffered. Table XVII shows the activity found in the two phases and the resulting distribution coefficient.

TABLE XVII

OCTADECANOL DISTRIBUTION DATA

Number	C H_2O	C octadecanol	$K = \frac{\text{C octadecanol}}{\text{C } H_2O}$
1	18.2	735.5	40.49
2	29.0	738.5	25.42
3	68.5	699.0	10.20
4	180.0	687.5	3.80
5	260.0	507.5	1.95

Inasmuch as these results are from unbuffered solutions, one might suggest this shift in distribution due to the pH effect resulting in the formation of a water soluble 8-hydroxyquinoline ion, probably formed from the ring nitrogen

A second experiment was carried out to determine the effect of the hydrogen ions produced by the reaction between $CuSO_4$ and 8-hydroxyquinoline on the partition

coefficient. Table XVIII shows the various pH after addition of $CuSO_4$ to a constant amount of 8-hydroxyquinoline. The pH was then adjusted to 5.0 and soybean oil was added as the second phase, mixed, and allowed to stand for several days.

TABLE XVIII

DISTRIBUTION DATA

Number	1	2	3	4	5	6
mM 8-hydroxyquinoline	.01	.01	.01	.01	.01	0.
mM $CuSO_4$	0.	.005	.01	0.1	1.0	1.0
Initial pH	6.80	3.30	3.28	3.29	3.72	4.20
Total Vol.	10.00ml	10.01ml	10.05ml	10.10ml	20.0ml	10. ml
Precipitation	0.	Heavy	Light	0.	0.	0.
Adjusted pH	5.00	5.00	5.00	5.00	5.00	5.00
Precipitation	0.	Heavy	Light	0.	0.	0.
Oil added	10 ml	10 ml	10 ml	10 ml	20 ml	20 ml

The soybean oil contained a considerable amount of free fatty acids, as evidenced by the characteristic green color in the copper-oil mixture alone. The stability of fatty acid copper salts is below that of 8-hydroxyquinoline, and thus no complication should be noted.

TABLE XIX

DISTRIBUTION DATA

(C in dps/ml)

Number	C H_2O	C oil	K = C oil/C H_2O
1	23.2	991.2	43.1
2	25.1	988.9	39.5
3	44.	970.0	22.1
4	58.2	955.8	16.5
5	178.	836.0	4.6

(Total activity added = 1014 dps)

D. Solubility in Aqueous Solution

From the experiments carried out, it now appears that the water solubility of copper-8-hydroxyquinoline may take place in two ways.

The first is by the following reaction:

(H^+ ion concentration constant)

$$CuQ_2 + \text{excess } Cu^{++} \rightleftharpoons 2CuQ^+$$

Water insoluble Water soluble

The second method is as follows:

$$CuQ_2 + \text{excess } H^+ \rightleftharpoons Cu^{++} + 2QH^+$$

Water insoluble Water soluble

The QH^+ ion is from the salt of the basic nitrogen in 8-hydroxyquinoline. Concentrated solutions of copper sulfate and 8-hydroxyquinoline form two types of crystals in H_2SO_4 solution that can be separated by macroscopic means. One is the copper sulfate, the other a yellow crystal that regenerates 8-hydroxyquinoline upon the addition of base to its water solution.

No salt of CuQ^+ has been prepared, but it should be possible if the correct anion were used.

The water solubility of copper-8-quinolinolate and 8-hydroxyquinoline was determined by saturating solutions with compounds of known specific activity.

The results were as follows:

(1) Copper (8-hydroxyquinoline)$_2$ - 4.44×10^{-6} Moles/liter (25°C)

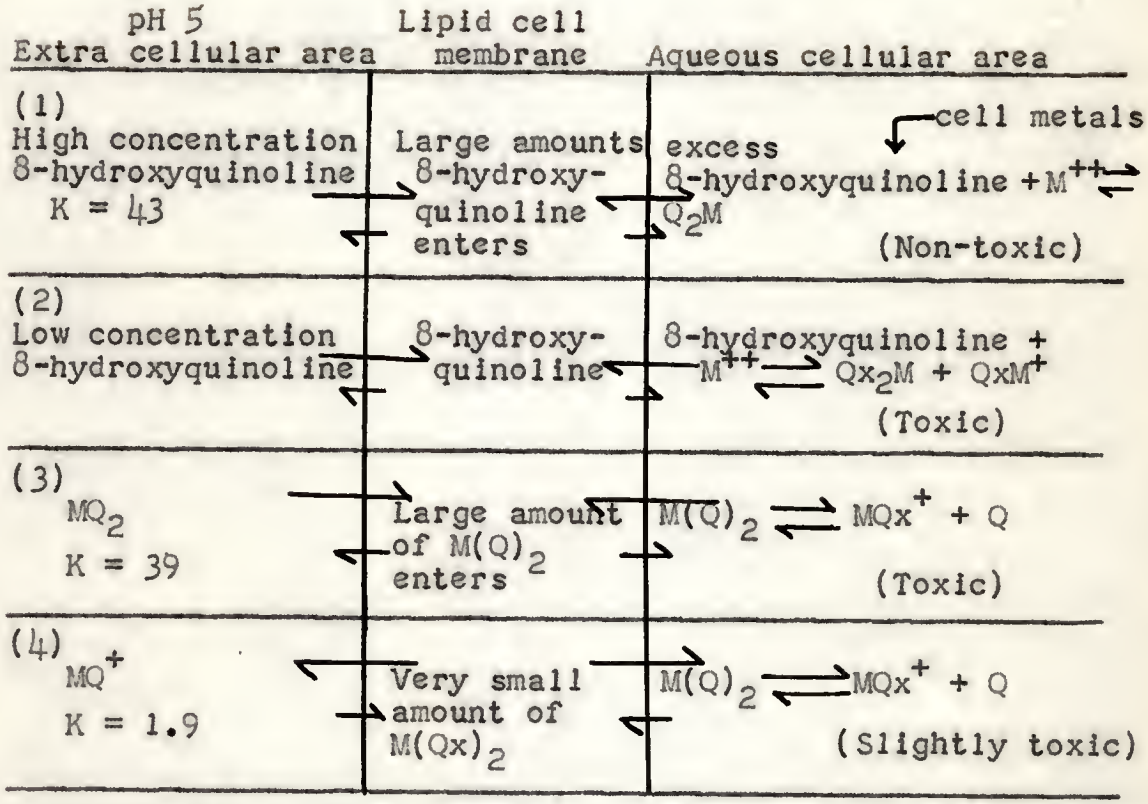
(2) 8-hydroxyquinoline - 1.38×10^{-3} Moles/liter (25°C)

VII. DISCUSSION OF RESULTS

The results from the experiments described establish certain fundamental facts as to the mechanism of action. Although discussion has been included throughout the previous sections, it would be appropriate at this time to review the data and consider the toxic action of 8-hydroxyquinoline or its copper chelate.

First, there can be little doubt that the action of this compound is taking place within the organisms themselves. This is borne out by the experiments with 8-hydroxyquinoline, by fractionation of spores and mycelia, by production of radioactive spores, and by radioactive mycelia. From the evidence at hand, one can say that the first action is to enter the cell. The ease of entrance, as has been shown, varies, depending on pH and form of the entering material. Copper-8-quinolinolate is lipid soluble, entering the cell easily with a partition coefficient of 43 at pH 5, while the water soluble CuQ^+ is unable to enter the cell (41). Free 8-hydroxyquinoline can enter the cell as well if not easier than its copper-8-quinolinolate.

Second, the information that some breakdown of the compounds within the cell is taking place. Further biological evidence, such as reversals with excess 8-hydroxyquinoline, reversals with excess copper ion, high toxicity at low concentrations, and low toxicity at high concentrations of 8-hydroxyquinoline, is powerful evidence that the toxic entities are neither the 2:1 complex nor free 8-hydroxyquinoline. The 1:1 complex, as proposed by Albert, must be considered to be the toxic ion. Its presence has been demonstrated by distribution and equilibrium experiments. One can now say that the toxic material is in the cell and that it is the 1:1 complex. It can further be said that the 8-hydroxyquinoline is being decomposed to CO_2. These facts can be diagrammatically represented as follows:



This gives a consistent picture of the mechanism of action up to the final step, that of just how the 1:1 complex is acting to disrupt the normal cell function. One may suggest that it is able to disrupt some important enzyme function by virtue of its oxidation potential or some other property that allows it to fit into the cells metabolism.

VIII. SUMMARY

Radioactive 8-hydroxyquinoline labeled in two different positions, the 9 position in one case and the 2,4 position in the other, was prepared for the first time. From these preparations it was noted that the preparation via labeled aniline-1-C^{14} resulted in a diluted product, suggesting that the reduced nitrobenzene enters the reaction and dilutes the product. This was not the case for the 8-hydroxyquinoline prepared via the labeled glycerol-1,3-C^{14}.

Many of the radioactive analyses were conducted using a modification of a new method of liquid counting. This method consists of making a solution of the radioactive material in a suitable solvent and counting the liquid in a special stainless steel cell. A solvent couple consisting of phosphoric acid-formamide (14 per cent phosphoric acid) was used as the solvent. The remainder of the counting was done by solid and gas methods.

The uptake of 8-hydroxyquinoline by A. niger mycelia was found to increase as the pH increased to 7. This effect could be predicted on the basis of the neutral molecule which is present in greatest amounts at pH 7.

Radioactive spores and mycelia were prepared by allowing the fungi to grow on 8-hydroxyquinoline-9-C^{14} media.

The uptake of 8-hydroxyquinoline by both spores and mycelia was measured. The spores were found to take up many times the amount taken up by the mycelia. The rate of uptake was very rapid for spores.

The uptake of copper-8-quinolinolate by both spores and mycelia was measured. The uptake was then determined for an excess of copper so the formation of the 1:1 chelate would be favored. It was found that water solubility was greatly increased on addition of excess copper. The distribution of the copper complex between water and various solvents showed the same increase in water solubility with increased copper concentration.

The solubility of 8-hydroxyquinoline and its copper complex was determined using labeled compounds.

A decomposition of the 8-hydroxyquinoline in contact with the living mycelia was found to occur in which some of the carbon-14 is converted to carbon dioxide.

A hypothesis as to how the 8-hydroxyquinoline or its metal complex is acting as a toxic agent is represented diagrammatically in the discussion section.

BIBLIOGRAPHY

(1) Hatta, S., Kitasato archives, 9, 1 (1932).

(2) Albert, A., Selective Toxicity, Methuen and Co., Ltd;, London, and John Wiley and Sons, Inc., N.Y., N.Y. (1951) pp 123.

(3) Albert, A., Med. J. Aust., i, 245 (1944).

(4) Albert, A., Magrath, D., Biochem J., 41, 534 (1947).

(5) Zentmeyer, G., Phytopath., 33, 1121 (1943).

(6) Zentmeyer, G., Science, 100, 294 (1944).

(7) Albert, A., Rubbo, S. D., Goldacre, R. J., and Balfour, B. G., Brit. J. Exper. Path., 26, 60 (1945).

(8) Albert, A., Gibson, M. I., and Rubbo, S. D., Brit. J. Exper. Path., 34, 119-130 (1953).

(9) Albert, A., Brit. J. Exper. Path., 36, 505 (1954).

(10) Vicklund, R. E., Manowitz, M. and Bagdon, V. J., Mycologia, 46, 133-141 (1954).

(11) Skraup, Z. H., Montas, 1, 317 (1880).

(12) Sidgwick, N. V., The Organic Chemistry of Nitrogen, Oxford University Press, London (1936), pp 544.

(13) Ruben, S., and Kamen, M. D., Phys. Rev., 59, 349 (1941).

(14) Reid, A. F., and Urey, U. C., J. Chem. Phys., 7, 137 (1943).

(15) Norris, L. D. and Snell, A. H., Science, 105, 265 (1947).

(16) Hollander, J. M., Perlman, I., and Seaborg, G. T., Rev. of Modern, Phys., 25, No. 2, 612-651 (1953).

(17) Berggren, J. L. and Osborne, R. K., Bull. Am. Phys. Soc. 23, No. 3, 46 (1948).

(18) Morgan, K. Z., Standards of Radiological Protection and Control, 23, Oak Ridge Summer Symposium (1951).

(19) Calvin, M., Heidelberger, C., Reid, J.C., Tolbert, B. M., and Yankwich, P.E., Isotopic Carbon, John Wiley and Son, Inc., N.Y., N.Y. (1949), pp 148-277.

(20) Van Slyke, D.D. and Folch, J.J., Biol. Chem., 136, 509 (1940).

(21) Schwebel, A., Isbell, H. S. and Karabinos, J. V., Science, 113, 465 (1951).

(22) Brownell, Land Lockhart, Nucleonics, 10, 26 (1952).

(23) Neville, O. K., J. Am. Chem. Soc., 70, 3501 (1948).

(24) Raaen, J. and Ropp, G.A., Anal. Chem., 25, 174 (1953).

(25) Wilzbach, Brown, and Kaplan, Science, 118, 522 (1953).

(26) Wilzbach, Van Dyken, and Kaplan, Anal. Chem., 26, 880 (1954).

(27) Palevsky, H., Swank, R. K., and Grenchik, R., Rev. Sci. Inst., 18, 298 (1947).

(28) Van Slyke, D. D., Mac Fayden, D. A., and Hamilton, P. J. Biol. Chem., 141, 671 (1941).

(29) Sutton, F., Volumetric Analysis, 12th Edition, Philadelphia, Penn. (1935).

(30) Kolthoff, I. M., and Stenger, V.A., Volumetric Analysis 2, 2nd Edition, John Wiley and Son Inc., N. Y., N. Y. (1947).

(31) Dauben, W. G., Reid, J. C., and Yankwich, P. E., Anal. Chem. 19, 828 (1947).

(32) Reid, A. F., Weil, A. S., and Dunning, J. R., Anal. Chem. 19, 824 (1947).

(33) Miller, McCallan and Weed, Contr. Boyce Thomp. Inst., 17, 151-171 (1953).

(34) Rubbo, Albert and Gibson, Brit. J. Exper. Path., 31, 425 (1950).

(35) Irving, H. and Williams, R. J., J. Chem. Soc. 3192 (1953).

(36) Meller, D. P., and Maley, L., Austral. J. Sci. Res., 2, 92 (1949).

(37) Johnston, H. and Frieser, D. F., J. Am. Chem. Soc., 74, 5239 (1952).

(38) Hutchison, A. W., et al, Solubility Behavior Physico-Chemical Nature, and Activity of Copper-8-Quinolinolate, Final Report to Quartermasters Research and Development Center, Watick, Mass. (1955).

(39) Glasstone, S., Text-Book on Physical Chemistry. Van Nostrand, N.Y., N. Y. (1940).

(40) Willard and Furman, Quantitative Analysis.

(41) Danielli, J. F., and Davson, H., J. Cell. and Comp. Physical, 5, 495 (1935).

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BIOGRAPHICAL ITEMS

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee. It was submitted to the Dean of the College of Arts and Sciences and to the Graduate Council and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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